The acquisition and maintenance of antibodies to merozoite antigens of Plasmodium falciparum and their role in protective immunity to malaria

Thesis

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The acquisition and maintenance of antibodies to merozoite antigens of *Plasmodium falciparum* and their role in protective immunity to malaria

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Abstract

Introduction: Measurement of antibodies at a single time point in malaria-endemic populations and associating them with protection and susceptibility has given insight into the development and maintenance of the immune response to malaria. Immunity to malaria is a dynamic process that takes place over a number of years in different stages, which implies that antibody measurement at a single time point may not adequately encompass acquisition of immunity.

Methods: Antibody responses to merozoite antigens (AMA1 and MSP2) were measured in 300 children aged 0-10 years twice a year for three years. Each child was followed up on a weekly basis for episodes of malaria. Factors affecting antibody responses at each time point and antibody contributions to protection from malaria were investigated, and the association between functional activity of anti-AMA1 antibodies and protection was assessed.

Results: Anti-AMA1 and anti-MSP2 antibody responses were dependent on the amount of prior and ongoing exposure and paralleled the decline in malaria transmission that occurred during the study period. High antibody responses were generated after 2-3 episodes of parasitemia and were likely to drop on further exposure. There was no evidence for polarisation of IgG responses to AMA1. The association between antibody responses and protection/risk of malaria was dependent on both the sample time and analysis method used. Antibody responses to AMA1(W2mef) were associated with an increased risk of malaria over the study period. Antibodies to MSP2(3D7) may be associated with protection from malaria. Antibodies to a functional invasion inhibitory epitope of AMA1(3D7) were rare but associated with protection from malaria.

Conclusion: Longitudinal assessment of antibody levels gives a more complete picture of acquisition and maintenance of malaria immunity. Antibodies can be both a measure of protection or susceptibility. These findings enrich knowledge on acquisition of immunity and will impact vaccination development and immunisation strategies.

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To my family – thank you for being both an inspiration and a challenge. Even though you sometimes didn't understand exactly what AMA1 and MSP2 were you were my biggest fans. Thank you.

And finally for the children experiencing malaria today – I hope that this work will contribute to reducing your burden.

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1 Introduction

1.1 The Burden of Malaria

More than 3 billion people live in areas where malaria transmission occurs and over 1 billion of them reside in areas that are at the highest risk of malaria (>1 reported case /1000/year). In Africa, where 12% of all those at risk of malaria reside, there were an estimated 212 million cases of malaria (90% of all cases worldwide) in 2006 which resulted in over 800,000 deaths, with most of the burden occurring in children younger than 5 years of age (WHO 2008). Over 75% of the cases of malaria in Africa were caused by *Plasmodium falciparum*. With only an estimated 1 in 5 malaria deaths reported worldwide, malaria plays a significant role in world health (WHO 2008).

Though most of the burden of disease due to *P. falciparum* is in Africa, malaria endemicity ranges from hypoendemic to holoendemic as a result of the vector distribution and climate. Up to 12% of surveys taken in a malaria transmission mapping study (Malaria Atlas Project, MAP) reported zero parasite prevalence and only 29% reported parasite prevalence above 50% (Guerra et al. 2008). Even with this great variation in malaria transmission across the continent, the disease has great economic and social consequences and is believed to contribute to the poorer development indices observed for endemic countries (Gallup and Sachs 2001; Sachs and Malaney 2002).

Transmission of *P. falciparum* malaria varies greatly across Africa, and impacts on the risk of infection and possible death early in life. Analysis from several sources indicates a wide range, from as low as a single infectious bite in a decade to several bites daily (Hay et al. 2000). Increasing malaria transmission intensity leads to increased clinical disease which is highest in areas of moderate malaria transmission intensity. Clinical disease manifests in younger children as malaria transmission rises and is seen in increasingly older individuals as malaria transmission decreases or becomes unstable (Snow et al. 1997; Modiano et al. 1998; Reyburn et al. 2005; Idro et al. 2006; O'Meara et al. 2008b; Okiro et al. 2009). This is due to the development of clinical immunity early on in life in areas of stable malaria transmission. The majority of infections of *P. falciparum* in malaria endemic areas are sub-patent, do not result in any clinical symptoms, and are

indicative of a degree of host immunity. Most of the clinical malaria that is seen is non-severe and manifests as non-specific febrile illness which is either cleared by the immune system or with the aid of anti-malarial therapy. Severe malarial syndromes (severe anaemia, impaired consciousness, metabolic acidosis, malaria in pregnancy) are the cause of a million deaths annually in Africa.

Africa appears to be undergoing a reduction in malaria transmission (observed by falling parasite prevalence rates and malaria hospital admissions) which has been attributed to large scale malaria control programmes. Interventions such as the use of effective anti-malarial therapeutics and insecticide treated bed nets (ITNs) have resulted in reducing both the burden of malaria and its associated mortality in Africa. Widespread use of ITNs in Kenya resulted in a 44% reduction in mortality children below 5 years over a two year period (Fegan et al. 2007). The combined use of ITNs and artemisinin-based combination therapy in Zanzibar reduced mortality by 52% in under-fives over a two year period (Bhattarai et al. 2007). The use of ITNs also affected malaria transmission dynamics and reduced parasite prevalence 10-fold in the same study. The continued use of these interventions has significantly reduced paediatric malaria admissions by up to 62% in parts of Kenya associated with intensive malaria control programmes (Okiro et al. 2007). Some of the reduction may be due to other factors such environmental and demographic changes.

1.2 The life cycle of *Plasmodium falciparum*

Malaria is caused by protozoan parasites of the genus *Plasmoduim* most of which are transmitted by mosquito vectors. All of the five main species of *Plasmodium* that naturally infect humans (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*) are transmitted by the female anopheline mosquito. All *Plasmodium* species causing human malaria have a complex life cycle that involves two stages of replication occurring in the vertebrate host and insect vector (Figure 1.1). Sexual replication occurs in the mid-gut of the insect vector and asexual reproduction in the human host. The vertebrate stage of the lifecycle is made up of exo-erythrocytic and erythroytic parts. Sporozoites injected by the biting mosquito enter the blood within an hour (Vanderberg and

Frevert 2004; Amino et al. 2006; Yamauchi et al. 2007) and infect hepatocytes (Mota et al. 2002; Pradel et al. 2002; Frevert et al. 2005; Baer et al. 2007). The sporozoites develop in the hepatocyte and generate numerous daughter merozoites per parent sporozoite within 14 days that are released at once into the circulation; destroying the host hepatocyte.

Invasion of erythrocytes is thought to occur soon after merozoite release from hepatocytes. The actual process of invasion is very quick (approximately 60 seconds) and will be discussed in detail in following sections. Within the erythrocyte, the merozoite differentiates into a flat ring form of the trophozoite stage and starts feeding on erythrocyte cytosol and haemoglobin (Aikawa et al. 1967; Langreth et al. 1978). During the mature trophozoite stage *P. falciparum* exports proteins to the surface of the erythrocyte that are ligands for receptors on host cells and leads to sequestration in various organs which protects the parasite from elimination by the spleen (Luse and Miller 1971; Langreth and Peterson 1985). By 38-40 hours post invasion the trophozoite has further differentiated into a schizont which divides to give 16-20 daughter merozoites (White and Kilbey 1996). At approximately 48 post invasion the distorted erythrocyte bursts and releases the merozoites into the circulation to continue the erythrocytic cycle. It is during the asexual part of the life cycle that the symptoms of malaria manifest.

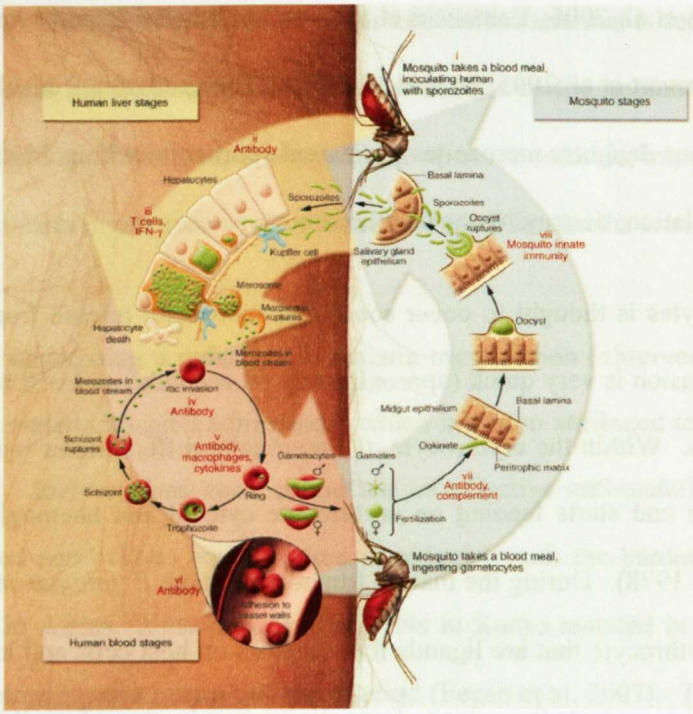

Figure 1.1 Life cycle of *Plasmodium* parasites showing the various stages that can be targeted by host immune responses. Human antibodies can act on newly injected sporozoites (i and ii), on merozoites (iv), and on parasite derived proteins on the surface of infected erythrocytes (v and vi). Immune cells and their related cytokines can act on infected hepatocytes and the infected erythrocytes (iii and v). In the mosquito vector antibodies and complement can act on the invasive ookinetes (vii), and innate immunity on newly released sporozoites (viii) (Greenwood et al. 2008).

Development of the initial sexual forms of the *Plasmodium* parasites also occurs during the erythrocytic stage. The decision for development into gametocytes occurs during formation of pigmented trophozoites (Bruce et al. 1990). The mechanisms involved in this differentiation are both genetic, with different *Plasmodium* isolates differing in their propensity to form gametocytes (Smalley et al. 1981; Alano and Carter 1990), as well as environmental (Price et al. 1996; Buckling et al. 1999; Talman et al. 2004). Mature gametocytes have reduced adhesive properties and are released into the circulation where uptake into the mosquito occurs during a blood meal.

1.3 *P. falciparum* merozoite invasion of erythrocytes

The merozoite form of *P. falciparum* is ovoid in shape with an apical prominence at one end (Langreth et al. 1978). The entire surface of the merozoite is covered in a 'fuzzy' coat of glycoslphosphatidylinositol (GPI) anchored proteins and peripheral proteins bound to other membrane-bound proteins (Sanders et al. 2005). Merozoites have a centrally placed nucleus, ribosomes, a mitochondrion, and a plastid. The apical end of the parasite contains organelles (micronemes, rhoptries, and dense granules) which secrete proteins that are involved in the invasion process. Several proteins located on the surface and in the apical organelles of the merozoite have potential roles in invasion (Figure 1.2). A number of these proteins have been characterised and will be discussed below.

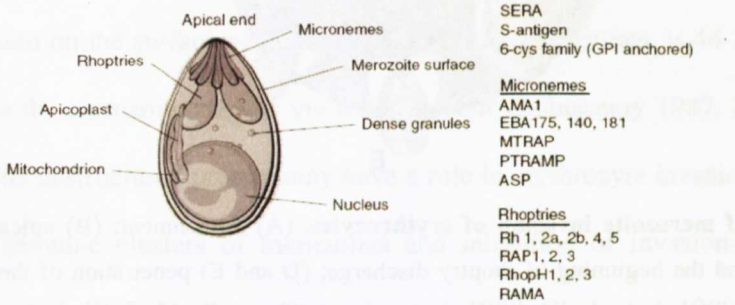

Figure 1.2 Structure and major antigens of the *P. falciparum* merozoite. The apical end of the merozoite has specific organelles involved in erythrocyte invasion. Surface proteins may be GPI anchored or associated with GPI-anchored proteins by molecular interactions. Listed are known proteins of the merozoite surface and organelles (Richards and Beeson 2009).

Prior to erythrocyte invasion, merozoites are released from the parent schizont in an explosive event that serves to disperse the merozoites (Glushakova et al. 2005). Several proteases are involved in the breakdown of the erythrocyte cytoskeleton and membrane, as well as the parasitophorous vacuole (Salmon et al. 2001; Miller et al. 2002; Hodder et al. 2003). The first contact that merozoites have with the erythrocyte appears to be a reversible, low affinity interaction followed by reorientation of the merozoite to ensure interaction between the apical end and the erythrocyte (Bannister and Dluzewski 1990). A non-reversible tight junction is then formed

between the erythrocyte membrane and the merozoite. This junction moves along the surface of the merozoite towards its posterior and permits entry of the merozoite into the erythrocyte. The movement of the tight junction is powered by an actin and myosin mechanism common to all apicomplexa (Soldati et al. 2004). Active entry into the erythrocyte results in shedding of the 'fuzzy' coat into the milieu by a serine protease (Harris et al. 2005b). The entire invasion process takes around a minute from initial attachment to full entry (Figure 1.3) (Gilson and Crabb 2009).

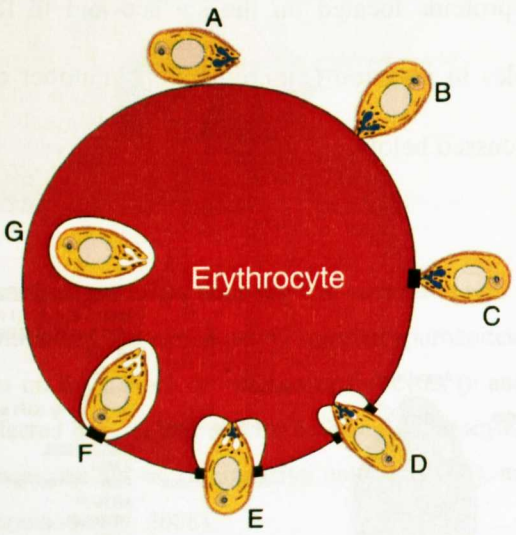

Figure 1.3 Stages of merozoite invasion of erythrocytes. (A) Attachment; (B) apical reorientation; (C) junction formation and the beginning of rhoptry discharge; (D and E) penetration of the merozoite past the tight junction into a forming parasitophorous vacuole; (F and G) pinching of the junction, and resealing of the red blood cell membrane. The surface coat of the merozoite is progressively stripped as it moves through the tight junction (D to F). The membrane surrounding the fully invaded merozoite is termed the parasitophorous vacuole membrane (Cowman et al. 2000).

Several merozoite proteins interact with receptors on the erythrocyte surface in each step of the invasion process. A full discussion on each of these proteins is beyond the scope of this review but a number of merozoite antigens will be described. The initial reversible attachment of the merozoite to the erythrocytes is thought to be facilitated by several interactions, but knowledge of these interactions is very limited. The best defined is the interaction between the most abundant surface antigen, merozoite surface protein 1 (MSP1), and receptors on the erythrocyte surface (Goel et al. 2003). MSP1 is one of a family of membrane associated proteins that are present on the merozoite surface. Following a proteolytic processing event at schizont rupture, MSP1 appears

on the surface of the merozoite as a GPI anchored complex made up of four fragments (Holder et al. 1992). On invasion of the erythrocyte another proteolytic event (during shedding of the fuzzy coat) results in cleavage of $MSP1_{42}$ into $MSP1_{19}$ which remains attached to the merozoite (Blackman et al. 1990; Blackman and Holder 1992). $MSP1_{19}$ contains two epidermal growth factor (EGF) -like domains that may have essential binding functions (Chitarra et al. 1999). Antibodies specific to $MSP1_{19}$ and $MSP1_{42}$ inhibit *in vitro* merozoite invasion (Blackman et al. 1990; Egan et al. 1999; Morgan et al. 1999; Darko et al. 2005). In spite of its abundance, MSP1 is not essential for parasite growth, as was seen by the successful replacement of *P. falciparum* MSP1 EGF-like domains with those from *P. berghei* MSP8 (Drew et al. 2004). $MSP1_{19}$ has been reported to bind to band 3, and recent studies have found that $MSP1_{42}$ binds to heparin-like molecules (Boyle et al. in press).

MSP2 will be discussed in detail later and will therefore be mentioned briefly here. MSP2 is abundantly expressed on the surface of mature schizonts and merozoites, is 44-55 kDa in size, and is also anchored to the merozoite surface via a GPI anchor (Ramasamy 1987; Epping et al. 1988; Smythe 1988). This unstructured protein may have a role in erythrocyte invasion as was shown by the formation of immune clusters of merozoites and inhibition of invasion by MSP2-specific antibodies (Epping et al. 1988; Miettinen-Baumann et al. 1988; Clark et al. 1989; Lyon et al. 1989). It appears to be essential as attempts to knock-out the gene have been unsuccessful (Cowman and Crabb 2006).

MSP3 is a 48 kDa protein that is the product of a proteolytic processing of a precursor protein and associates with other proteins on the merozoite surface (Oeuvray et al. 1994; LaCount et al. 2005). MSP3 may have a role in erythrocyte invasion as parasites with a truncated form of the protein invade erythrocytes less efficiently (Mills et al. 2002).

MSP4 is a 40 kDa polymorphic membrane anchored protein (Marshall et al. 1997). MSP4 has 53% homology with sporozoite and liver stage antigen (SALSA) which is involved in the invasion of hepatic cells. Antibodies to peptide sequences of SALSA have also recognised MSP4 on the

merozoite surface (Puentes et al. 2004). A single high activity binding peptide (HABP) of MSP4 that binds to erythrocytes has low invasion inhibition activity and indicates that this protein may be involved in the initial steps of erythrocyte recognition (Garcia et al. 2007).

MSP6 is a 48 kDa dimorphic protein that forms a complex with MSP1 and MSP7 on the merozoite surface (Trucco et al. 2001; Pearce et al. 2004). Antibodies induced in rabbits by immunisation with recombinant MSP6 and MSP6-derived HABP inhibit merozoite invasion *in vitro* suggesting that this protein has a role in invasion (Pearce et al. 2004; Lopez et al. 2006). MSP7 is a highly conserved 41 kDa protein and is part of the above mentioned complex on the merozoite surface (Pachebat et al. 2001). Disruption of the *msp7* gene was reported to slow parasite growth and lead to preferential invasion of reticulocytes (Tewari et al. 2005).

Serine repeat antigen (SERA) is a highly conserved protein that undergoes proteolytic processing to shorter fragments that are associated with the merozoite surface (Li et al. 2002b; Sato et al. 2005). Early studies showed that monoclonal antibodies specific to SERA inhibited merozoite invasion *in vitro* (Barr et al. 1991; Bathurst et al. 1993). SERA-specific antibodies have also been shown to act with monocytes via antibody dependant cellular inhibition (ADCI) mechanisms to inhibit parasite growth (Pang and Horii 1998; Soe et al. 2002).

Another group of antigens with possible roles in parasite invasion are found in the apical organelles (micronemes and rhoptries) of the merozoite. Apical membrane antigen 1 (AMA1) is the most studied of these and will be discussed in detail later in this chapter. Briefly, AMA1 is an integral membrane protein that is initially localised to the micronemes of the merozoite but spreads to the merozoite surface after schizont rupture. AMA1 is thought to be involved in the apical re-orientation of the merozoite, and possibly tight junction formation just before invasion occurs (Alexander et al. 2006; Baum et al. 2008).

After re-orientation the invasion process begins and involves interactions between proteins secreted from the apical organelles and receptors on the erythrocyte membrane. The erythrocyte binding

antigens (EBA) have a Duffy binding-like (DBL) domain and are contained in the micronemes and include EBA140, EBA175, EBA181, and EBL1 (Camus and Hadley 1985; Gilberger et al. 2003b; Mayer et al. 2004; Mayer et al. 2009). EBAs bind to sialic acid on the erythrocyte surface but can utilise alternative receptors for erythrocyte invasion (Duraisingh et al. 2003b; Stubbs et al. 2005). EBA175 is a 175 KDa soluble protein that is found in the supernatant after schizont rupture (Sim et al. 1992). Although EBA175 is involved in parasite invasion, gene disruption does not inhibit this process thus showing that it is not essential for parasite invasion (Kaneko et al. 2000; Reed et al. 2000; Duraisingh et al. 2003a) because of a degree of redundancy between the different EBAs and PfRh proteins. Interestingly, antibodies specific to EBA175 are able to inhibit merozoite invasion *in vitro* (Orlandi et al. 1992). EBA181 is likely to play a similar role to that of EBA175. Disruption of the gene has no detectable effect on parasite invasion (Gilberger et al. 2003a). EBA140 is not expressed by all *P. falciparum* strains and although it plays a role in invasion, it is not required for invasion and growth of parasites (Thompson et al. 2001). EBA140 binds to glycophorin C receptor on erythrocytes (Lobo et al. 2003).

Rhoptry proteins are thought to be released onto the merozoite membrane where they play important roles during invasion. The reticulocyte binding protein homologue family (PfRh) includes PfRh1, PfRh2a, PfRh2b, PfRh4, and PfRh5 which are variably expressed by the merozoite (Rayner JC 2001; Duraisingh et al. 2003b; Stubbs et al. 2005; Triglia et al. 2005; Baum et al. 2009). The Rh proteins bind to various receptors on the erythrocyte surface so their variable expression may be a way for the merozoite to utilise different invasion pathways (Duraisingh et al. 2003b; Stubbs et al. 2005) and evade immune responses (Persson et al. 2008). This family of proteins are related to reticulocyte binding proteins in *P. vivax* which allow the merozoites to preferentially invade reticulocytes rather than mature erythrocytes (Galinski et al. 1992; Galinski and Barnwell 1996; Galinski et al. 2000). They are also related to the p235 rhoptry proteins in *P. yoelii.* Interestingly, merozoites from the same schizont can express different members of these proteins which may be a mechanism for immune evasion (Preiser et al. 1999).

Rh1 binds to a sialic acid-dependent trypsin-resistant receptor on the erythrocyte membrane referred to as receptor Y (Rayner et al. 2001). Anti-PfRH1 antibodies inhibited merozoite invasion of trypsin treated erythrocytes which helped define a trypsin independent pathway of merozoite invasion (Rayner et al. 2001). Two erythrocyte HABPs of PfRH1 also had invasion inhibitory activity *in vitro* (Valbuena et al. 2003). Though important in invasion, Rh1 is not essential for parasite survival as the gene has been successfully knocked out in *P. falciparum* parasites (Triglia et al. 2005). Rh2a and Rh2b are proteins more than 350 kDa in size with identical amino acid sequences apart from the C-terminal region (Rayner et al. 2000; Triglia et al. 2001). Both Rh2a and Rh2b genes can be knocked out and have no effect on parasite survival although Rh2b is required for interaction with receptor Z which is sialic-acid independent (Triglia et al. 2001; Duraisingh et al. 2003b). Although non-essential, these proteins are involved in invasion as antibodies specific to both have inhibited invasion of 3D7 parasites (Triglia et al. 2001). Rh4 has been shown to be essential for invasion pathway switching via differential expression and is responsible for sialic acid-independent invasion (Stubbs et al. 2005). Antibodies to Rh4 can also inhibit invasion (Tham et al. 2009).

Rhoptry associated protein 1 (RAP1) is found in a non-covalently bound complex (called QF3) with RAP2 and RAP3 on the merozoite surface (Clark et al. 1987; Ridley et al. 1990). Although monoclonal antibodies to RAP1 and RAP2 inhibit invasion, merzozoites with truncated RAP1 proteins can successfully invade erythrocytes (Schofield et al. 1986; Harnyuttanakorn et al. 1992; Baldi et al. 2000). All the proteins mentioned above and numerous others that are likely to have roles in merozoite invasion of erythrocytes indicate the complexity of this process.

1.4 Immunity to blood-stages of *P.falciparum* infection

Due to the focus of this thesis, this review of immunity to malaria will focus only on *P. falciparum* rather than other human malarias, and primarily focus on human studies rather than animal models. Infection with *P. falciparum* in naïve individuals may be clinically significant the first time. Only a

few individuals develop significant clinical symptoms even with continuous exposure to the parasite. Natural non-sterile immunity is acquired in these individuals as evidenced by the presence of measurable parasites in their blood and a lack of clinical symptoms. This non-sterile immunity is a combination of various immunological responses to infection. Clinical immunity is defined as the acquired response that protects against disease in spite of the presence of parasitaemia and protects against mild or severe disease. Anti-parasite immunity is the acquired response that severely inhibits growth and replication of parasites or completely eliminates them from the individual, or prevents the infection of hepatocytes. Clinical immunity develops earlier than anti-parasite immunity. Reduction in severe disease incidence precedes the reduction of parasitaemia by several years (Marsh and Snow 1997). In areas of moderate to high transmission, after the initial susceptibility to severe malaria, children often develop clinical immunity by the age of five. At this point they still have high parasite burdens. At the population level, this immunity to severe non-cerebral malaria is generated after 2-3 episodes of disease (Gupta et al. 1999). Anti-parasite immunity appears to be more difficult to generate and less efficacious in its nature. Therefore immunity to malaria develops in three stages, with protection from severe disease generated much earlier than that to mild disease, and finally, only partial immunity to parasitisation *per se*.

Innate and adaptive immune responses are required for the establishment of both clinical and anti-parasite immunity to *P. falciparum* malaria. Repeated infection of non-immune individuals shows that the peak parasitaemia reached in an individual is independent of parasite strain or species (Molineaux et al. 2002). This could be due to the induction of the innate immune system and is supported by further studies showing that the mechanisms of innate immunity are triggered when parasite density reaches a particular threshold (Kwiatkowski and Nowak 1991; Bruce and Day 2003). These mechanisms limit the maximum parasite density in a stage and strain independent manner until the adaptive immune mechanisms can then eliminate the parasites (Richie 1988).

1.4.1 Innate immune responses to infection

The innate immune response is the first line of protection against all invasive organisms and is carried out by the coordinated efforts of several cell types that are triggered by molecules characteristic of microbial pathogens. Monocytes are found in the peripheral circulation before migration into tissues and differentiation into macrophages. In these localities macrophages are involved in the general clean up of end products resulting from normal cellular activity as well as the destruction of extracellular and intracellular pathogens. Most macrophages are found in the spleen where they play a role in the removal of altered or aged erythrocytes. Macrophages are activated by pro-inflammatory cytokines and in this state can activate memory T cells as well as promote B cell differentiation. Natural Killer (NK) cells are lymphocytes with natural cytotoxic activity that do not require activation by any other cell type in order to act. Their activity is regulated by a balance in the expression of inhibitory and activating receptors through which inducible molecules, pathogen markers, or stress signals may be relayed. Natural Killer T (NKT) cells are a line of cells that have the characteristics of both NK and T cells. They are found in the periphery as well as the spleen and thymus and are particularly abundant in the liver. NKT cells are directly cytotoxic to virus infected cells (Skold and Behar 2003) and could act in an immunoregulatory manner in the activation of T cells (Hansen et al. 2003). γδT cells are found in the gut and mucosal tissues of adults. They are a small percentage of circulating T calls but may play a part in immunity to intracellular pathogens such as *Plasmodium* (Dieli et al. 2001). Dendritic cells are classic antigen presenting cells that reside in all tissues of the body. They continuously screen their environment and are activated on interaction with foreign antigen or cytokine release from infected cells. On activation they migrate to the lymph nodes and spleen where they trigger a cascade of activation and maturation steps in cells of the adaptive immune system. All these cell types respond to *Plasmodium* infection in model systems as well as natural infection in humans.

Plasmodium infected erythrocytes bind to CD36 that is expressed on macrophages leading to their phagocytosis in vitro (Serghides et al. 2003). Other in vitro studies show that macrophages release

TNF-α when exposed to infected erythrocytes (Scragg et al. 1999; Hensmann and Kwiatkowski 2001). Even at sub-patent levels of infection, in vivo studies show elevated levels of NK cell activation markers (Hermsen et al. 2003). These cells produce IFN-γ soon after encountering infected erythrocytes in vitro (Artavanis-Tsakonas and Riley 2002). NKT cells also produce IFN-γ, but later than NK cells (Artavanis-Tsakonas et al. 2003). γδT cells are activated by infected erythrocytes and their numbers in the peripheral blood increase during acute disease (Hviid et al. 2001) and the frequency of these cells is twice as high in healthy Ghanaian adults compared to their European counterparts (Hviid et al. 2000). γδT cells also inhibit growth of blood-stage *P. falciparum* parasites when in contact with the infected erythrocyte (Troye-Blomberg et al. 1999). Infected erythrocytes have been shown to modulate the function as well as maturation of dendritic cells (Urban et al. 1999). These cells have a reduced ability to produce Il-12 and cannot activate T cells. Dendritic cells that have derived from monocytes that have phagocytosed haemozoin are unable to properly respond to maturation signals which will also have an effect on downstream activation of effector cells (Skorokhod et al. 2004). The innate immune response is therefore essential for limitation of the initial round of parasite replication and giving the host time to mount an adaptive immune response.

1.4.2 Adaptive immune responses to infection

Naturally acquired immunity to malaria is dynamic and appears in those individuals with exposure to the infective *Plasmodium* parasite. The earliest known evidence for acquired immunity to malaria came about by examination of blood smears by Robert Koch from individuals living in high and low malaria endemicity settings in Java in the late 1800s when he observed that control of parasite density came sometime after immunity from clinical disease. This was confirmed some decades later (McGregor et al. 1956). Later studies showed that acquired immunity to malaria occurs in two main phases. Anti-disease immunity protects against morbidity and appears to be acquired relatively quickly early on in life followed by anti-parasite immunity which protects against high parasite densities but not total parasite clearance (Marsh and Snow 1997; Gupta et al.

1999). Sterilising immunity to malaria is probably rarely achieved or sustained, and adults in endemic areas are often asymptomatic carriers of *Plasmodium*. Population indices of immunity to malaria in an area of mesoendemic transmission in Kenya shows that immunity to severe disease is achieved by the age of five followed by immunity to mild disease in children younger than 12 years and eventual, though non-sterile parasite immunity in adults (Marsh 1992; Marsh and Kinyanjui 2006) (Figure 1.4).

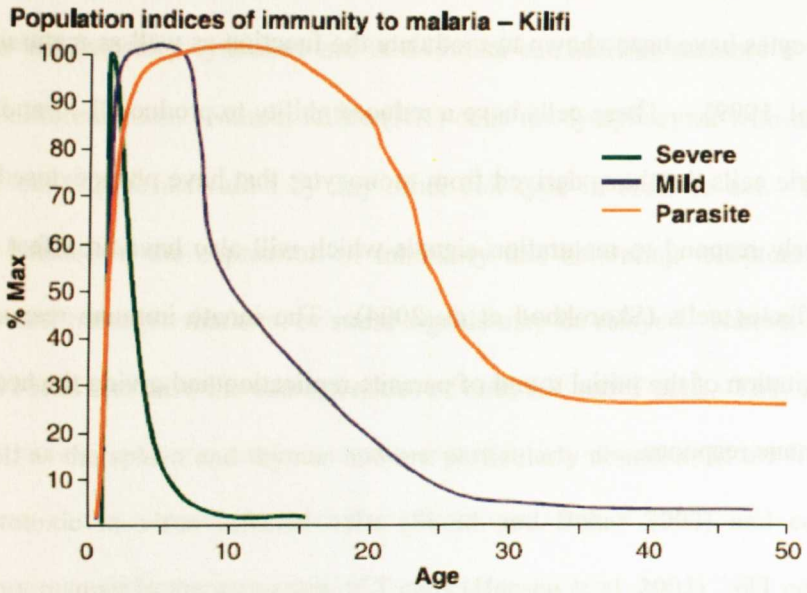

Figure 1.4 Population indices of immunity to malaria – Kilifi. The figure shows representative data from a number of studies in Kilifi District on the coast of Kenya. The age pattern of asymptomatic parasite prevalence and the period prevalence of both mild and severe clinical malaria are shown in relation to maximum prevalence recorded (Marsh and Kinyanjui 2006).

1.4.2.1 Cell-mediated immunity to malaria

Cell-mediated responses play an important role in protective immunity to malaria but due to the focus of this thesis will only be discussed briefly. Early evidence for cell-mediated immunity came from murine malaria models that demonstrated the development of malaria immunity in the absence of antibodies. Mice which had their B cell development inhibited or were genetically deficient in B cells and had previously experienced parasite challenge managed to mount a

secondary immune response but were unable to completely clear their infections (Grun and Weidanz 1983; van der Heyde et al. 1994; von der Weid et al. 1996). Further evidence came from the adoptive transfer of both splenic cells and T cells in the absence of antibodies to naïve mice which led to their protection from parasite challenge (Spitalny et al. 1977; Egan et al. 1987). A possible mechanism for the action of cell-mediated immunity in the absence of antibodies would involve an interaction between antigen presenting cells and CD4+ T cells leading to CD4+ T cell activation and the resulting activation of macrophages and production of effector molecules (Good 2001). Implication of CD8+ T cells in murine immunity came from studies where CD8+ T cells were depleted from immune mice and resulted in loss of immunity (Schofield et al. 1987; Weiss et al. 1988).

In humans, a role for both CD4+ and CD8+ T cells in immunity to malaria has been suggested in a number of studies. Malaria exposed human CD4+ T cells can proliferate and release IFN-γ on exposure to a range of pre-erythrocytic and erythrocytic antigens (Zevering et al. 1992; Ndungu et al. 2006). The presence of CD4+ T cells that are specific to a conserved sequence in circumsporozoite protein (CSP) were predictive of protection from both malaria infection and disease in The Gambia over 4 months (Reece et al. 2004). CD8+ T cells, which are found in naturally exposed populations as well as vaccinated individuals, have also been associated with protection from severe malaria in children (Hill et al. 1991). Immune cells secrete both anti-inflammatory and pro-inflammatory cytokines and the balance of these cytokines can contribute to the various malaria syndromes as well as their possible outcomes, as has been seen in studies carried out in Vietnam and Gabon on adults and children (Day et al. 1999; Perkins et al. 2000). Down-regulation of IFN-γ has been observed in individuals living in an area of high malaria-transmission of Ghana compared to those living in an area of low-malaria transmission in the same country (Rhee et al. 2001). The authors suggest that clinical immunity (as would be achieved by individuals in high malaria-transmission settings) may be associated with the down-regulation of pro-inflammatory cytokines and thus a reduction in the symptoms of malaria.

1.4.2.2 The role of antibodies in protection against malaria

Antibodies have been shown to play a significant role in protection against malaria in both animal models and human studies. The earliest evidence for the protective effect of antibodies was produced by experiments carried out in the Gambia where purified immunoglobulin from immune adults was transferred to children suffering from clinical malaria (Cohen et al. 1961). Eight out of 12 children (aged 4 – 30 months) managed to clear their parasitaemia. A later study endorsed the earlier findings by showing the protective effect of purified African IgG on clinical symptoms as well as parasitaemia in eight Thai patients (Sabchareon et al. 1991). Naturally occurring transfer of antibodies from pregnant mothers to the foetus via the placenta also suggested a protective effect of maternal antibodies from clinical disease and parasite density in infants below 6 months (Hogh et al. 1995; Branch et al. 1998).

The three main mechanisms by which antibodies may carry out their protective role are; (a) inhibition of invasion of merozoites into erythrocytes, (b) inhibition of adhesion of parasitised erythrocytes to endothelial walls, and (c) antibody-immune cell interactions such as antibody-dependent cellular inhibition (ADCI), neutrophil-mediated killing, and opsonic phagocytosis.

1.4.2.2.1 Invasion Inhibition

Inhibition of invasion was put forward as a possible mechanism of antibody action in 1970 when the addition of immune serum to *P. knowlesi* cultures did not affect parasite growth but reduced reinvasion of parasites (Cohen and Butcher 1970). *In vitro* inhibition activity was further demonstrated by immune serum from convalescent Gambian children and healthy Papua New Guinean adults (Wilson and Phillips 1976; Brown et al. 1982). Agglutination of merozoites by monoclonal antibodies specific to merozoite antigens also led to a marked reduction in invasion (Epstein et al. 1981). Possible mechanisms of invasion inhibition suggested are inhibition of binding of merozoite proteins to erythrocyte receptors, agglutination of merozoites or inhibition of processing of those antigens essential for invasion (Cohen and Butcher 1970; Blackman et al. 1994;

Dutta et al. 2003). The effectiveness of invasion inhibitory antibodies was also reduced by the action of naturally occurring antibodies that block the action of invasion inhibitory antibodies to MSP1 (Guevara Patino et al. 1997; Nwuba et al. 2002).

Though inhibition of invasion was shown to be dose dependent in early studies, higher antibody titres were not always predictive of invasion inhibition by sera from different malaria endemic regions of the Philippines (Cohen and Butcher 1970; Sy et al. 1990). Inhibition of invasion by monoclonal and polyclonal antibodies specific to antigens involved in invasion such as MSP1 (Chappel and Holder 1993; Rotman et al. 1998), AMA1 (Hodder et al. 2001), EBA175 (Narum et al. 2000a; Rayner JC 2001), and Rh2b (Duraisingh et al. 2003b) showed the effective specific action of antibodies though results were variable.

The observation that actual values of invasion inhibition (reported as the percentage growth compared to a control culture) could vary widely even when using the same sera led to growth inhibition assay (GIA) technique standardization (Persson et al. 2006). This high throughput method produced results that were more consistent from smaller sample volumes. The high throughput method allowed for population studies to be carried out where the relationship between invasion inhibition and protection from clinical malaria could be investigated. It was hypothesised that the amount of functional antibodies would increase with age and exposure. High levels would likely be associated with protection from clinical malaria. Two studies carried out on samples from Kenya show that invasion inhibition activity appears highest in children and decreases as children get older (Dent et al. 2008; McCallum et al. 2008). Although high inhibitory activity was related to a modest delay in time to infection in one study, there was no reduced risk of symptomatic malaria in the other study (Dent et al. 2008; McCallum et al. 2008). These studies indicate that invasion inhibition may play a more significant role when the parasite burden is relatively high (childhood) but may not contribute much to protection against subsequent clinical malaria episodes.

1.4.2.2.2 Adhesion Inhibition

Cytoadherence of parasitised erythrocytes to endothelial cells of capillaries and the downstream associated effects are the cause of severe malaria syndromes. *P. falciparum* sequesters in organs in order to evade clearance from the host via the spleen. During the mid-late trophozoite stage, the parasite inserts proteins into the erythrocyte cell membrane that facilitate adhesion to endothelial cells. These variant surface antigens (VSAs) – of which *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) is the best known – bind to numerous receptors on host cell surfaces including CD36 and inter-cellular adhesion membrane protein 1 (ICAM-1).

VSAs undergo clonal antigenic variation and utilize this antigenic switching to evade the host immune response and maintain chronic infections (Brown et al. 1968; Butcher and Cohen 1972). Up-regulation and expression of certain *var* genes is associated malaria pathogenesis including severe childhood malaria and pregnancy associated malaria (Salanti et al. 2003; Deitsch and Hviid 2004; Jensen et al. 2004; Warimwe et al. 2009). VSAs are thought to be major targets of protective immune responses as blocking their action is thought to have a direct effect on the pathogenesis of malaria. Several studies have shown that individuals rarely have antibodies to the VSAs expressed by the infecting isolates but these are quickly acquired after infection, and subsequent disease episodes will be caused by parasites with VSA types that are not already recognized by the individual (Marsh and Howard 1986; Bull et al. 1998; Giha et al. 1999; Ofori et al. 2002). Anti-VSA antibodies that inhibit the adhesion of parasitised erythrocytes to chrondoitin sulphate A (CSA) on placental cells have been detected in multigravidae women who appear more able to induce this response faster than primigravidae women (Fried et al. 1998; Ricke et al. 2000; O'Neil-Dunne et al. 2001; Costa et al. 2006). This action is thought to be one of the mechanisms that protect against placental malaria. Anti-VSA antibodies also promote clearance of parasitised erythrocytes by opsonic phagocytosis (Tebo et al. 2002; Keen et al. 2007; Feng et al. 2009).

1.4.2.2.3 Antibody-dependent cellular inhibition

Antibody-dependent cellular inhibition (ADCI) involves an interaction of the Fc region of antibodies with receptors on monocytes that induces destruction of the opsonised foreign. It has also been suggested that monocytes release soluble factors that also inhibit parasite growth. Several *in vitro* studies carried out in the 1980s showed that ADCI is a significant part of the antibody dependent response to *P. falciparum*. Phagocytosis of merozoites and parasitised erythrocytes by moncytes from malaria patients was seen only in the presence of hyper-immune sera from malaria patients, whereas non-immune sera could not induce the phagocytosis of parasitised or non-parasitised erythrocytes (Khusmith et al. 1982). The ability of monocytes from non-sensitised individuals to phagocytose merozoites and parasitised erythrocytes with increasing efficiency as sera from increasingly immune individuals was added cemented the role of antibodies in ADCI (Khusmith and Druilhe 1983). A co-operative effect between non-sensitised monocytes and immune sera on reduction of *P. falciparum* growth was also noted (Khusmith et al. 1982). Antigen targets of antibodies involved in ADCI include MSP3 and GLURP (Oeuvray et al. 1994; Theisen et al. 1998). These studies show a clear role for anti-malaria antibodies in ADCI.

1.4.3 Antibody maintenance in malaria

Antibody levels to merozoite antigens are short-lived and appear dependent on the level of malaria transmission in the area. This has been shown in several studies including one in which antibody levels to RAP1 were measured in individuals living in areas with differing malaria endemicity (Gambia, Indonesia, Kenya, and Sudan). Antibody levels increased with increasing malaria endemicity (Jakobsen et al. 1997). More recently, a study carried out in Kenya measuring IgG1 and IgG3 levels to MSP1, MSP2, AMA1, and EBA175 in convalescent children showed that antibody levels dropped significantly 12 weeks after an episode of moderate to severe malaria requiring hospital admission (Kinyanjui et al. 2007). In areas of seasonal malaria transmission, antibody levels appear to be maintained as long as parasite exposure continues. Several studies comparing the antibody response in adults and children during wet (high transmission) and dry

(low transmission) seasons consistently support this observation (Fruh et al. 1991; Ramasamy et al. 1994; Cavanagh et al. 1998; Soares et al. 1999b; John et al. 2002). The drop in antibody prevalence to several antigens including MSP1, MSP2, CSP, and liver stage antigen 1 (LSA1) ranged from 11% over five months in Kenya up to 50% over three months in Sri Lanka (Ramasamy et al. 1994; John et al. 2002).

As antibody prevalence is positively associated with higher malaria transmission, the findings that antibody levels are higher in parasitised individuals does not come as a surprise. Empirical evidence for this was shown when the antibody levels to *P. vivax* MSP1 in individuals whose patent parasitaemia was treated by use of anti-malarial drugs dropped when the infection was cleared (Soares et al. 1997). A further study showed an average 13-fold drop in antibody levels to MSP1 following treatment (Soares et al. 1999a). Other studies on MSP2 also showed that antibody prevalence was associated with presence of parasites though there was only a significant increase in the levels of parasitised compared to non-parasitised children rather than in adults (Al-Yaman et al. 1994).

Not all antibody responses are short-lived; there appears to be a long-lived antibody response that can be maintained in adults. Two studies in the Gambia showed no significant change in adult antibody levels to MSP1 and MSP2 over a period of up to two years although children exhibited fluctuating levels of antibodies during the dry and rainy seasons over a period of three years (Riley et al. 1993; Taylor et al. 1996). Antibodies to schizont and crude sporozoite extract were shown to persist in adults from Senegal and Burkina Faso up to 11 years after they had settled in Paris (Druilhe et al. 1986). Initial exposure to *Plasmodium* does not need to occur over a long period of time. A single *P. vivax* outbreak that occurred in 1988 in a non-endemic region in Brazil was followed by intensive anti-malaria therapy that cleared all parasitaemia in the population. Without any subsequent exposure to malaria, antibodies to MSP1 could still be detected in 47% of individuals after 7 years (Braga et al. 1998).

When taken into account, most evidence points to a relatively short-lived antibody response that is rapidly initiated on subsequent exposure to antigen. But the fact that some antibodies can be detected in some individuals long after exposure to malaria is indicative of sustained antibody secretion by plasma cells. Antibodies may be released by long-lived plasma cells which occupy a niche in the spleen and bone marrow (Slifka and Ahmed 1998; Manz et al. 2002) or sub-patent persistent infection could also re-stimulate memory B cells (Ochsenbein et al. 2000; Bernasconi et al. 2002). The evidence for immune memory against malaria is limited. But interestingly, small numbers of memory B cells to MSP1, AMA1, and CIDR1α have been detected in individuals living in a malaria-endemic area (Dorfman et al. 2005).

Contrary to the response to malaria infection, natural or vaccine-induced antibody responses to some other infections appear to be long-lived. Antibody titres to measles have a half life of 12 years and antibodies can still be detected using a plaque reduction neutralisation antibody assay up to 33 years after vaccination (Dine et al. 2004). The long-lived antibody response to tetanus toxoid also appears to be maintained by the effective establishment of a pool of long-lived plasma cells as well as memory B cells. In the United Kingdom the triple vaccine for diphtheria, tetanus, and pertussis (DTP) is given at 2-4 months and at 4-5 years; and the tetanus and low dose diptheira vaccine (Td) given at 15-19 years as a booster. Even without the booster dose at 15-19 years, protective levels of antibodies to tetanus toxoid could be detected in 53% of individuals over 60 years of age (Maple et al. 2000). In Kenya tetanus immunisation occurs as a single dose in infancy with few opportunities for boosting either by immunisation. Antibodies to tetanus toxoid could still be detected in 71% of Kenyan individuals tested (Dorfman et al. 2005). Memory B cells specific to tetanus toxoid are present in vaccinated individuals (Lanzavecchia et al. 1983; Leyendeckers et al. 1999; Nanan et al. 2001).

1.4.4 Haemoglobinopathies and resistance to malaria

Sickle cell and α+ thalassemia are two main haemoglobinopathies affecting the incidence of malarial disease in Africa. Sickle cell is caused by a structural variant S of haemoglobin which

results in the adoption of a characteristic sickle shape by erythrocytes. Alpha (α+) thalassemia is characterised by low production of normal α-globin chains during haemoglobin production. Early observations that the prevalence of the sickle cell trait (HbAS) overlapped with areas of high malaria transmission led to the hypothesis that HbAS individuals had a selective advantage against their normal (HbAA) counterparts with regards to malaria outcome. Case control studies in West and East Africa established that indeed, HbAS does confer a considerable resistance to severe and complicated malaria (Willcox et al. 1983; Aidoo et al. 2002). More recently, it was shown that although HbAS had no effect on asymptomatic malaria, it was almost 90% protective against severe or complicated malaria in children below the age of five and resulted in parasite loads more than 4-fold less than in HbAA children (Williams et al. 2005b).

The immunity conferred by HbAS is in part due to the physical nature of the erythrocytes although immune regulation has also been implicated. Up-regulation of cell-mediated responses to malaria antigens in individuals from Sudan along with increased recognition of parasite infected erythrocytes in Gambian children was the initial evidence for immune regulation in HbAS individuals (Marsh et al. 1989; Bayoumi et al. 1990; Abu-Zeid et al. 1992). HbAS may regulate both the innate and adaptive arms of immunity. This was investigated in a study in Kenya which showed that protection from clinical malaria increased with age at a more rapid pace in HbAS children than in HbAA children, thus implying a role for HbAS is regulation of the adaptive immune response (Williams et al. 2005a).

Epidemiological studies in Melanesia suggested that α+ thalassemia is selected for by malaria based on the altitude- and latitude-dependent correlation between *P. falciparum* prevalence and the frequency of α+ thalassemia in the region (Flint et al. 1986; Yenchitsomanus et al. 1986). Studies in Papua New Guinea and Kenya showed that homozygotes for α+ thalassemia were 60% more protected than normal individuals from severe malaria (Allen et al. 1997; Williams et al. 2005d). Both these studies also showed that heterozygotes were also 40% more protected than normal individuals. A smaller study in Ghana showed the same protective effect in heterozygotes but not in homozygotes (Mockenhaupt et al. 2004). Although inhibition of parasite growth and invasion have been suggested as mechanisms for the protective action of α+ thalassemia, this seems unlikely

as the parasite densities measured in the Kenyan study were no different in homozygotes and normal individuals (Williams et al. 2005d; Wambua et al. 2006). Another study in Papua New Guinea also suggested that mechanisms other than inhibition of parasite growth or invasion confer this protection as they observed the same parasite densities as seen by Williams et al and Wambua et al (Fowkes et al. 2008). Interestingly, when both haemoglobinopathies are inherited together, their individual protection from severe malaria is lost. Children with HbAS and homozygous for α+ thalassemia were at the same risk of severe malaria as baseline in a study carried out in Kenya which may be explained by negative epistasis (Williams et al. 2005c).

A number of other haemoglobinlopathies have been associated with protection from malaria, but will not be summarised here in the interests of space.

1.4.5 The effect of exposure on immunity to malaria

The distribution of malaria morbidity and mortality is dependent on malaria transmission intensity. When hospital admissions due to malaria are analysed a pattern of rapidly increasing admission rates with increasing transmission that saturates as transmission intensity rises is observed, and may even decline in areas of hyperendemicity (Snow 1987; Trape et al. 1987; Marsh and Snow 1999). Numerous studies, including some carried out in Burkina Faso, Uganda, and Tanzania indicate that the mean age that children present with symptomatic malaria is consistently lower in areas of high transmission (Modiano et al. 1998; Reyburn et al. 2005; Idro et al. 2006). The median age of children presenting at hospital was 1 year in a high transmission setting compared to 3 years and 5 years in medium and low transmission settings (Reyburn et al. 2005).

In a recent longitudinal study that analysed malaria rates over 16 years in an area with changing malaria transmission intensity the mean age of children experiencing clinical malaria increased as malaria transmission decreased (O'Meara et al. 2008b). Although the incidence of cerebral malaria initially increased over the study period, the overall rates of malaria mortality declined due to a

decrease in the rates of severe malarial anaemia (O'Meara et al. 2008a). These results agree with earlier findings that the prevalence of severe malaria syndromes is partly dependent on transmission intensity. Presentation of cerebral malaria is higher in areas of low transmission intensity whereas severe malarial anaemia is more prevalent in high transmission settings and is exhibited by younger children than those presenting with cerebral malaria (Marsh and Snow 1999).

In areas of differing malaria endemicity there is a significant linear relationship between the intensity of exposure as measured by the entomological inoculation rate (EIR) and both prevalence and density of *P. falciparum* infection (McElroy et al. 1994; McElroy et al. 1997; Beier et al. 1999). Higher density parasitaemia was observed during high transmission seasons compared to low transmission seasons although no overall change in parasite prevalence was seen from season to season (Trape et al. 1993; McElroy et al. 1994). In a low transmission setting in Senegal, incidence of primary episodes of clinical malaria peaked during the high-transmission season in children between 7 and 11 years of age (Trape et al. 1993). Severe malaria is observed even in areas of very low transmission intensity though the risk in childhood remains lowest in higher transmission settings (Mbogo et al. 1993; Mbogo et al. 1995).

A large study in Congo that involved 500,000 study participants residing in areas of different transmission intensity indicated that large differences in malaria exposure may affect severe malaria rates slightly (Trape et al. 1987). This was confirmed in later studies that investigated malaria morbidity and mortality in infants and children living in areas of differing malaria transmission. By examination of hospital admission records in Kenya and Tanzania it was established that although the prevalence of severe disease was similar in both settings, the risk was higher in children below the age of one when in high transmission settings but this fell dramatically over the ensuing 10 years of life (Snow et al. 1994). In the comparable low transmission setting, risk of severe disease became higher in the second year of life and remained so over the next ten years.

Further observations in five settings of variable transmission intensity confirmed that the mean age of malaria disease decreased with increasing transmission intensity (Snow et al. 1997). In high transmission settings the risk of severe malaria declined significantly after 6 months of age compared to those in low transmission settings where the risk rose after five months and remained high throughout infancy (Snow et al. 1998). In both Congo and Kenya, the incidence of severe malaria was the lowest in children with the highest malaria exposure (Trape et al. 1987; Snow et al. 1997). These studies suggest that clinical immunity develops at different rates dependent on transmission intensity. Children living in a high transmission setting are exposed to malaria earlier and more frequently which leads to development of clinical immunity at a younger age.

1.4.6 The effect of age on immunity to malaria

The resistance to clinical malaria and infection that is exhibited by adults living in hyper- and holo-endemic areas of malaria transmission is thought to be due to the cumulative exposure over many years to malaria resulting in strong strain-specific and a degree of strain-transcending immunity. Another hypothesis is that the immunity exhibited by adults over children may be contributed by intrinsic host factors that are attributable to normal development of the host and immune system.

Observations in areas of differing malaria transmission intensity also point to an age-dependent factor in immunity to severe disease. Over various transmission settings in Africa there is a trend of reducing incidence of severe anaemia as children approach their fifth birthday whilst incidence of cerebral malaria increases with age (Snow et al. 1994; Imbert et al. 1997; Snow et al. 1997; Modiano et al. 1998). Two studies in children below 6 showed that as children got older parasite prevalence dropped, and although they were at less risk of high density parasitaemia increasing age was not predictive of reduced incidence of parasitaemia (Beadle et al. 1995; McElroy et al. 1997). Peak parasitaemia in high transmission settings occurs in children at about five years of age and declines in an age-dependent manner (Marsh et al. 1995). The same pattern is seen in low transmission settings but with the peak occurring at an older age.

The most convincing evidence for the age-dependent hypothesis comes from studies on transmigrant populations in Irian Java. A re-settlement scheme of the Indonesian government from densely populated non-malaria endemic islands to less populated malaria-endemic islands was the setting for a natural experiment for the effect of age on malaria immunity (Baird et al. 1991; Baird et al. 1993; Hudson Keenihan et al. 2003; Keenihan et al. 2003). Here, it was possible to separate the effect of age and exposure as the transmigrants were a relatively naive population with 20 months exposure that could be age-matched and directly compared to that of the native population that had experienced life-long exposure.

Comparison of parasite prevalence revealed that although overall parasite prevalence was higher in the transmigrant population, both populations exhibited a decrease in prevalence as age increased (Baird et al. 1991). Also, although overall time to first infection was shorter in the transmigrant population than the native one, the time to first infection increased with age in both populations in all age groups thus implying that the benefit of life-long exposure was no different in children and adult natives. In malaria endemic populations there is a drop in parasite prevalence as age increases which is attributed to immunity due to cumulative exposure to malaria. In the first year, parasite prevalence in the transmigrant population of did not decrease with age although 16 to 24 months later a pattern of decreasing parasite prevalence with increasing age became apparent (Baird et al. 1993).

On initially moving to malaria-endemic Irian Java, 95% of 240 transmigrants tested did not have measurable antibodies to ring-infected erythrocyte antigen (RESA). 20 months later, not only could antibodies to RESA be detected in the transmigrant population, sero-positivity significantly increased with age (Baird et al. 1991). This pattern was similar to that in the native population which, in that population would have been indicative of long term cumulative exposure to malaria. Glycosyphosaphatidylinositol (GPI) anchors are the major carbohydrate modification in erythrocytic *P. falciparum*, and are thought to be released into the circulation during schizont rupture, and elicit an immune response in malaria exposed individuals. Anti-GPI antibodies in the transmigrant population were measured at enrolment and after every malaria episode for up to 8 episodes over 3 years. Adults had significantly raised anti-GPI antibodies after a single infection of

malaria that remained consistently high and did not rise after further infections (Hudson Keenihan et al. 2003). In children anti-GPI antibodies rose significantly after each infection though their responses remained significantly lower than those of adults after the first three infections. Over the course of all infections, adults were 4 times more likely to have a high antibody response to GPI (Hudson Keenihan et al. 2003).

In an extension of this study, antibody responses to pre-erythrocytic (CSP and TRAP) and erythrocytic (EBA175, MSP1-19, MSP4, RESA, and schizont extract) *P. falcipaprum* antigens were measured after each infection. Across all the infections observed, adults were significantly more likely to mount an antibody response to CSP (OR=1.8), TRAP (OR=4.2), RESA (OR=3.0), schizont extract (OR=2.5%), MSP1-19 (OR=1.8), MSP4 (OR=2.7), and EBA175 (OR=20.1), although prevalence of the response to EBA175 was lower than for the other erythrocytic antigens in both adults and children, and may be explained by the limited time that EBA175 is exposed to the immune system due to its apical organelle localisation in the merozoite (Keenihan et al. 2003).

A separate study carried out in Kenya has also produced evidence for the age-dependent immunity hypothesis. The relationship between pubertal development and resistance to *P. falciparum* over two transmission seasons was investigated in males between the ages of 12 and 34 years (Kurtis et al. 2001). All study participants were tested for parasites before being cleared of parasitaemia at the beginning of each transmission season and then monitored for frequency and density of new infections over the transmission season. Individuals with negative blood slides at the beginning of the seasons before treatment were on average 3 years older than those with positive blood slides. The development of resistance was investigated before, during, and after puberty. Resistance to *P. falciparum* infection increased with age during puberty but not before puberty, and the frequency of parasitaemia decreased with age after puberty. Increased levels of pubertal hormones DHEAS (a measure of adrenarch) and testosterone (a measure of gonadarche) also predicted resistance to infection independent of age (Kurtis et al. 2001).

Adults and children also appear to be at different risks of severe disease during their primary exposure to *P. falciparum*. During the first six months of a transmigrant community's exposure to

malaria the adult transmigrants had a 4.5-fold higher risk of clinically diagnosed severe malaria than their children (Baird et al. 1998). In low transmission settings malaria pathology appears at lower parasite densities and the risk of disease at any parasite density is higher in older individuals (Petersen et al. 1991; Smith et al. 1994). Studies on naive returned travellers have also mentioned the higher severity of disease in older individuals; two will be mentioned here. Out of 135 returned travellers in Israel with *P. falciparum* infection, those ≥ 40 years were at higher risk of developing severe malaria (OR=4.29) compared to those < 40 years old (Schwartz et al. 2001). Another study looking at malaria in returned travellers in Italy, there was a significant increase in the prevalence of severe malaria (3% to 24%) with increasing age (< 30 years to ≥ 50 years) (Calleri et al. 1998).

1.5 Two vaccine candidates in review: Apical membrane antigen 1 (AMA1) and merozoite surface protein 2 (MSP2)

AMA1 and MSP2 are merozoite proteins that are leading malaria vaccine candidates and will be discussed in detail below. Their progress as vaccines will be discussed later in this chapter.

1.5.1 Apical Membrane Antigen 1 (AMA-1)

1.5.1.1 Location and structure of AMA1

AMA1 was first identified in *P. knowlesi* as an invariant merozoite antigen to which monoclonal antibodies inhibited the in vitro invasion and growth of homologous parasites (Deans et al. 1982; Deans et al. 1984; Thomas et al. 1984). AMA-1 is present in all Plasmodium species (Marshall et al. 1989; Waters et al. 1990; Kappe and Adams 1996; Kocken et al. 2000) and is synthesised during the late schizont stage as an 83kDA precursor protein that is localised to the micronemes of the merozoite (Deans et al. 1984; Bannister et al. 2003). The N-terminal prosequence of the 83kDa protein is cleaved in the micronemes and the mature 66kDa form of AMA1 is then translocated on

to the merozoite surface (Howell et al. 2001; Healer et al. 2002; Howell et al. 2003; Howell et al. 2005). Further processing of the 66kDa form occurs on the surface of the merozoite prior to erythrocyte invasion. The protein is cleaved into 44-48kDa soluble forms by the membrane-bound subtilisin-like PfSUB2 sheddase which leaves a small remnant of AMA1 on the merozoite surface (Howell et al. 2003; Harris et al. 2005b; Howell et al. 2005).

AMA1 is a type I integral membrane protein that varies between 556 and 622 amino acids in length, with *P. falciparium* AMA-1 being 622 amino acids in length and only 50 of these making up the cytoplasmic domain (Chesne-Seck et al. 2005). The ectodomain is made up of three domains (DI, DII, and DIII) (Figure 1.5) and an N-terminal prosequence that are held in a tertiary structure by 8 conserved disulphide bonds (Hodder et al. 1996). Numerous interactions between domains occurs resulting in the 'folding over' of the N-terminal end of DI towards the merozoite surface by interaction with the other two domains (Pizarro et al. 2005). DI and DII are more structured than DIII and contain folding motifs of the plasminogen-apple-nematode (PAN) domain family. That the PAN domain is associated with receptor binding function implicates AMA1 in the initial steps of erythrocyte invasion. Both DI and DII contain loop sections with a 40 amino acid conserved loop in both domains and unstructured loops in DI (Pizarro et al. 2005).

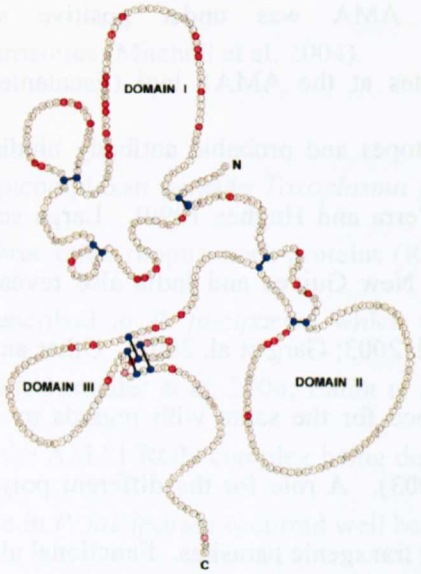

Figure 1.5 Schematic of the *P.* falciparum AMA1 ectodomain, showing the three separate domains, I, II and III. The locations of eight disulphide bridges are shown in blue. Residues shaded in red and magenta represent mutations in different naturally occurring P. falciparum sequences (Nair et al. 2002).

1.5.1.2 Polymorphisms of AMA1

AMA1 is encoded on a single gene and is highly polymorphic (Thomas et al. 1990b; Chesne-Seck et al. 2005). AMA1 appears to be essential for parasite survival as attempts to knock out its gene have not been successful (Triglia et al. 2000). It has 64 polymorphic positions that are spread throughout its sequence with 32 in DI, 11 in DII, and 9 in DIII. The pro-sequence contains 9 polymorphisms and the cystolic region 3 (Chesne-Seck et al. 2005). Only one position (Glu197) is heptamorphic, positions 200 and 201 are tetramorphic, and the rest either di- or trimorphic (Bai et al. 2005).

The crystal structure of AMA1 reveals a long hydrophobic region in DI that is hypothesised to be a ligand-binding site. It is surrounded by a region consisting of the highest number of polymorphic residues in the protein (Bai et al. 2005). These polymorphisms appear to have arisen due to diversifying selection in order to avoid invasion-inhibitory antibodies or other protective immune responses (Cortes et al. 2003; Polley et al. 2003). Invasion inhibitory epitopes in domains II and III have also been described (Nair et al. 2002; Mueller et al. 2003; Collins et al. 2007).

Polymorphisms in AMA1 are thought to be a result of parasite evasion from host immune pressure. Early studies showed that AMA was under positive selection as shown by the synonymous/nonsynoymous rates at the AMA1 loci (Escalante et al. 1998). A later study confirmed that both T-cell epitopes and probable antibody binding sites on AMA1 were under positive balancing selection (Verra and Hughes 1999). Large scale genetic analyses in malaria endemic populations in Papua New Guinea and India also revealed that DI was under positive balancing selection (Cortes et al. 2003; Garg et al. 2007). Other analyses on populations in Nigeria and Thailand also gave evidence for the same with regards to domains II and III (Polley and Conway 2001; Polley et al. 2003). A role for the different polymorphisms in AMA1 has been suggested by experiments using transgenic parasites. Functional allelic replacement of domain I of AMA1 resulted in differing susceptibilities of *P. falciparum* strains to growth-inhibitory antibodies thus suggesting a functional role for the polymorphisms in AMA1 (Healer et al. 2004).

A recent study showed that AMA1 variants worldwide could be grouped into six distinct genetic clusters (Duan et al. 2008). A later study that contained a greater number of *ama1* sequences from different locations confirmed the six subgroups and their worldwide distribution as defined in the smaller Duan *et al* study (Barry et al. 2009), although another study has found a much large number of clusters using this approach (Takala and Plowe 2009).

1.5.1.3 Function of AMA1

The successful invasion of murine erythrocytes by transgenic *P. falciparum* parasites containing the complete *P. chabaudi* ectodomain was strong evidence for the role of AMA1 in invasion of erythrocytes (Triglia et al. 2000). A study that measured the levels of invasion of parasites with chimeric (*P. falciparum* and *P. chabaudi*) AMA1 proteins in the presence of anti-*Pf*AMA1 immunoglobulins showed that all three extracellular domains are involved in interactions with erythrocyte surface molecules (Healer et al. 2005). Merozoites can only initiate invasion of an erythrocyte via their apical end. Clear evidence for the exact role of AMA1 in invasion was provided by ultrastructural analysis. Whilst anti-AMA1 monoclonal antibodies allowed the initial attachment of *P. knowlesi* merozoites to the erythrocyte membrane, they inhibited full invasion by impeding reorientation of the merozoites (Mitchell et al. 2004).

Host cell invasion by another apicomplexan parasite *Toxoplasma gondii* involves a complex that includes *T. gondii* AMA1 and three other rhoptry neck proteins (RON2, RON4, and RON5). An ortholog of RON4 has been described in *P. falciparum* which associates with AMA1 and is localised to the moving junction (Alexander et al. 2006; Baum et al. 2008). RON2 was recently described in *P. falciparum* with the AMA1/RON complex being detected in mature schizonts thus indicating that complex formation in *P. falciparum* occurred well before invasion (Cao et al. 2009).

To date, only one possible receptor for AMA1 on the erythrocyte surface has been suggested. DIII of recombinant *Pf*AMA1 binds to the receptor Kx on trypsin-treated erythrocytes, but cannot bind

trypsin-treated Kx_{null} erythrocytes (Kato et al. 2005). Although parasites can still invade Kx_{null} erythrocytes, it is at a significantly reduced rate, which further implicates Kx as a receptor for AMA1. Domains I and II of AMA1 also have a role in the interaction between merozoite and erythrocyte as shown by the successful adhesion of COS7 cells expressing *P. yoelii* DI and DII to mouse and rat erythrocytes (Fraser et al. 2001). Interestingly, a role for AMA1 in the invasion of hepatocytes by sporozoites has also been suggested. *Pf*AMA1 is expressed in sporozoites where it undergoes proteolytic processing similar to that in the merozoite. *Pf*AMA1 is no longer expressed after the sporozoite has invaded the hepatocyte and anti-AMA1 immunoglobulins as well as serine protease inhibitors that inhibit shedding of AMA1 both inhibit sporozoite infectivity (Silvie et al. 2004).

Recent generation of *P. falciparum* GFP-tagged AMA1 parasites indicate that AMA1 could have two distinct roles in the invasion process that are dependent on the short cytoplasmic domain of the protein (Treeck et al. 2009). This study showed that even though parasites expressing AMA1 with heterologous EBA175 or Rh2b cytoplasmic domains had correctly trafficked AMA1, they were unable to invade erythrocytes whereas those with homologous cytoplasmic domains from *P. vivax* and *P. berghei* could functionally invade. Complete invasion also required the correctly phosphorylated cytoplasmic domain. Non-phosphorylated mutant parasites were able to correctly reorient themselves but could not invade thus implicating AMA1's two-step involvement in merozoite invasion of erythrocytes (Treeck et al. 2009).

1.5.1.4 AMA1-specific invasion inhibition

Antibodies to AMA1 are able to inhibit invasion of erythrocytes by merozoites *in vitro* (Hodder et al. 2001; Healer et al. 2004), and although most of these inhibitory antibodies are targeted to polymorphic epitopes, and inhibitory antibodies are generally strain-specific, there is some evidence that invasion-inhibitory antibodies could also recognize conserved or less polymorphic epitopes (Kennedy et al. 2002; Collins et al. 2007). Inhibition of erythrocyte invasion by parasites appears to be variant specific as well as cross reactive. Antibodies produced by the immunisation

of rabbits with recombinant 3D7 *Pf*AMA1 significantly inhibited invasion of *P. falciparum* field isolates and lab strains that share sequence similarity (eg. D10) whereas those antibodies generated by immunization with recombinant FVO *Pf*AMA1 were able to inhibit erythrocyte invasion by field isolates as well as lab strains with divergent AMA1 sequences (Duan et al. 2008). Though both animal and human antibodies to AMA1 can inhibit invasion of erythrocytes, most of the antibodies tested interact with strain-specific epitopes in order to exert this functionality (Hodder et al. 2001). Interestingly, AMA1 specific antibodies appear to have higher invasion inhibitory activity than $MSP1_{42}$ specific antibodies which could be indicative of this antigen's important role in invasion (Miura et al. 2009). Antibodies to AMA1 appear to exert their invasion inhibitory effects via two main actions. The first is by cross-linking and trapping the final soluble 44-48kDa AMA1 fragments on the surface of the merozoite (Dutta et al. 2003). The other mechanism involves inhibition of secondary processing of 66kDa AMA1 and its subsequent redistribution on the merozoite surface (Dutta et al. 2005). Recent evaluation of antibody responses in children living in a malaria endemic are identified that Senegalese children with high antibody responses to AMA1(FVO) also had higher growth inhibitory activity than those with lower antibody responses although there was a age related decline in this activity (Courtin et al. 2009).

Several monoclonal antibodies to AMA1 can also inhibit invasion *in vitro*. Monoclonal antibody (mAb) 1F9 is strain specific and inhibits invasion of 3D7 and D10 *P. falciparum* parasites but not HB3 or W2mef, and recognises a polymorphic epitope in domain I (Coley et al. 2001; Coley et al. 2006). The D10 and 3D7 strains of AMA1 are identical in domain I but differ in domains II and III. The actual binding site of mAb 1F9 appears to be part of the hydrophobic trough and the surrounding loops on the AMA1 surface. This interaction between AMA1 and mAb 1F9 is quite large, conformationally dependent, and substitution of residue 197 completely inhibits the AMA1-1F9 interaction (Coley et al. 2006). The interface between AMA1 and 1F9 is made up of a large buried area that the antibody protrudes into. Though this interface is unusual, binding of mAb 1F9 does not significantly affect the structure of AMA1 (Coley et al. 2007). Antibodies from exposed individuals from Papua New Guinea compete with mAb 1F9 for binding to AMA1, whereas antibodies from non-malaria exposed individuals do not.

Another invasion inhibitory monoclonal antibody that has been studied in detail is 4G2. Initial studies showed that 4G2 exerted its inhibitory effects not by simple steric effects but by binding to a functionally important part of AMA1. This same study revealed that the mAb 4G2 epitope was conserved across all *P. falciparum* variants and was situated in the domain II loop of AMA1 (Collins et al. 2007). AMA1 forms a complex with the essential rhoptry neck protein RON4 which then associates with the moving junction during invasion. MAb 4G2 appears to exert its inhibitory effects by binding to the domain II loop before the AMA1/RON4 complex forms and therefore inhibits downstream invasion actions (Collins et al. 2009).

Monoclonal antibody 5G8 is not strain specific in its interactions with *P. falciparum* (Coley et al. 2006). It recognizes a 19 residue linear epitope in the N-terminal pro-sequence/pro-domain of AMA1 (Coley et al. 2001).

In an attempt to investigate binding of AMA1 to its receptor, phage display technology was used to identify novel peptides that bound to AMA1. A 15-mer peptide (F1) that bound to *Pf*AMA1 alone and inhibited invasion of human erythrocytes was identified. Further investigation identified its epitope as the same one as that of mAb 4G2 (Li et al. 2002a). Following the success of this method, another phage library containing peptides that bound to mAb 4G2dc1 was investigated and three reactive peptides (J1, J3, and J7) were identified. All the peptides elicited an immune response from rabbits that recognized AMA1, and both J1 and J7 were recognized by human plasma from Papua New Guinea (Casey et al. 2004). *In vitro* invasion inhibition by antibodies specific to J1 and J7 from human and rabbit palsma was also observed. A fourth 20-mer peptide (R1) has also been identified that binds to the same epitope as F1 and elicits a stronger invasion inhibitory effect (Harris et al. 2005a). It appears that all the mentioned peptides and epitope-specific antibodies bind to a 'hot spot' on AMA1 and exert their invasion inhibitory effects in the same manner.

1.5.1.5 Vaccine potential of AMA1 in animal models

Most of the evidence for AMA1 as a potential target for protective immune responses has come from vaccination/challenge animal studies. In all the studies, the use of correctly folded AMA1 for vaccination, followed by challenge with a homologous parasite strain, resulted in varying levels of protection in all the animal models used (Deans et al. 1988; Collins et al. 1994; Anders et al. 1998; Narum et al. 2000b; Salvatore et al. 2002; Stowers et al. 2002; Burns et al. 2003; Burns et al. 2004). Passive transfer of anti-AMA1 specific mAb into BALB/c mice followed by challenge with *P. yoelii* led to controlled sub-patent parasitaemia and survival of all the mice (Narum et al. 2000b).

In a study where five rhesus monkeys were vaccinated with *P. vivax* and challenged with *P. cynomolgi*, antibody titres to *Pv*AMA1 were boosted and there was a slight reduction in parasitaemia, but the animals were not protected from subsequent infection from *P. cynomolgi* (Kocken et al. 1999). Another study where BALB/c mice were vaccinated with *P. chabaudi* DS AMA1 and challenged with *P. chabaudi* 556KA AMA1 – which differs from the DS variant at 79 sites – resulted in no reduction in parasitaemia (Crewther et al. 1996). These two vaccination studies showed that protection was as a result of a response to homologous rather than heterologous parasites. More recently, immunisation of rabbits with a mixture of PfAMA1 alleles (3D7, HB3, and FVO) led to proportionally more antibody responses to conserved epitopes but also had the same functional capacity to inhibit homologous parasite invasion as antibodies generated by single allele immunisation (Kusi et al. 2009). Though earlier studies indicated that most antibodies to AMA1 were to strain specific epitopes, this one suggested that there is a large portion of functional antibody responses to conserved epitopes that may be optimally induced by exposure to multiple AMA1 alleles. This finding was interpreted by the authors as good evidence for a multi-allele single antigen vaccine approach.

Though some of the overall antibody response to AMA1 is to conserved regions of the molecule, as seen by the high concordance between antibody levels to various AMA1 alleles and demonstrated

using competition ELISA with different AMA1 alleles, there is also a significant portion of the antibody response that is strain-specific (Hodder et al. 2001; Polley et al. 2004; Cortes et al. 2005). Currently the extent to which antibodies target conserved versus strain-specific epitopes and their relevance to immunity is not clear. Passively transferred rabbit antibodies induced by vaccination with *Pc*AMA1 DS did not reduce parasitaemia in mice challenged with *P. chabaudi* 566KA whereas they significantly reduced parasitaemia in mice challenged with the homologous parasite strains (Crewther et al. 1996). Also, the antibodies elicited by rabbit vaccination with AMA1 3D7 and antibodies induced by natural infection from Papua New Guinea which have been purified on recombinant AMA1 3D7 selectively inhibit invasion of *P. falciparum* 3D7 compared to *P. falciparum* HB3 (Hodder et al. 2001).

Up to half of the overall antibody response can be strain-specific. This was seen in one animal study where vaccination with AMA1 3D7 or AMA1 FVO induced two-fold higher antibody titres to the homologous strain (Kennedy et al. 2002). Sera from immunised animals in the same study also showed higher inhibitory activity towards parasites of the homologous strain. This strain-specific invasion inhibition has also been observed in another study which measured invasion of *P. falciparum* FCR3 and NF54 parasites (Kocken et al. 2002).

1.5.1.6 Naturally acquired antibodies to AMA1

AMA1 is highly immunogenic and this is seen by its high recognition by sera from malaria endemic populations. Several studies have reported above 65% antibody prevalence to various alleles (3D7, HB3, D10, FVO, 7G8) of AMA1 in populations from Africa and Australasia (Thomas et al. 1994; Johnson et al. 2004; Polley et al. 2004; Cortes et al. 2005). Antibodies to DI were more prevalent than those to DII and DIII (Polley et al. 2004; Cortes et al. 2005); however, it is unclear whether domains expressed separately are correctly folded.

Antibody titres to AMA1 increased with age in all the studies mentioned earlier except for one where although there was an increase in antibody titres with age in one population, it was not the

case for the other population studied (Thomas et al. 1994). These differences could be down to differences in methodology or antigen (the AMA1 protein used in the Thomas et al study was highly glycosylated, which is not the case for native AMA1). Antibodies also appear to be acquired faster and earlier in areas of high transmission compared to low transmission, and antibody titres are generally higher in parasitemic individuals (Polley et al. 2004). There is also some evidence for the transfer of maternal antibodies to AMA1 across the uterus with more than 50% of newborns tested being positive for anti-AMA1 antibodies (Riley et al. 2000; Metenou et al. 2007).

Strain-specific and cross-reactive antibodies to AMA1 are detected in malaria endemic populations. There are detectable responses to polymorphic epitopes measured by ELISA and invasion inhibition assays (Hodder et al. 2001; Polley et al. 2004; Cortes et al. 2005). Antibodies to polymorphic epitopes appear to dominate in childhood with the frequency of allele-specific responses decreasing significantly with age (Cortes et al. 2005). Anti-AMA1 antibodies in malaria endemic populations are predominantly IgG1 and IgG3 with very little IgG2 or IgG4 detected (Riley et al. 2000; Polley et al. 2004; Metenou et al. 2007; Nebie et al. 2008; Stanisic et al. 2009). Although titres of both IgG1 and IgG3 increased with age and exposure to malaria, IgG1 responses predominated over IgG3 responses both in prevalence and intensity (Riley et al. 2000; Nebie et al. 2008; Stanisic et al. 2009).

1.5.1.7 The role of anti-AMA1 antibodies in protection from clinical disease

Several immuno-epidemiological studies have been carried out in order to establish whether antibodies to defined malaria antigens are associated with protection from malaria. They usually involve measurement of antibodies at baseline with extended passive or active follow-up. The results from these studies are not always clear and may conflict with each other. The role of antibodies to AMA1 in protection from malaria is no different. To date, there are published studies

that both support an association with protection as well as other studies that give evidence for the opposite.

Three studies, two of them carried out in the same part of Kenya; provide evidence that antibodies to AMA1 are associated with protection. Anti-AMA1 antibodies to the 3D7 and FVO variants were associated with protection from clinical malaria in children and adults over a period of 6 months. This protection was seen only for those individuals who were parasite positive at the time of sampling (Polley et al. 2004). A later study on samples from the same area on children showed that high levels of antibody reactivity was associated with a reduced risk of hospital admittance over 8 months (Osier et al. 2008). Although antibodies to AMA1 were predictive of protection in this study; it was only when anti-AMA1 antibodies were analysed in combination with those to MSP2 or MSP3 that the protection was significant. A similar result had also been obtained from a study carried out in The Gambia. Here, using a microarray immunoassay, antibody reactivity to both AMA1 and MSP2 in combination was more predictive of protection with the individuals in this group only experiencing asymptomatic malaria (Gray et al. 2007).

A slight trend towards protection was associated with high anti-AMA1 antibodies but was not significant in another study carried out in a high transmission setting in Kenya. In this study, any parasitaemia was cleared by treatment at baseline and then individuals were followed up for 12 weeks and the point of re-infection monitored. High antibody levels to AMA1 were not associated with a longer time to re-infection (John et al. 2005). Though high antibody titres to MSP3 were associated with protection over a 6 year period in Dielmo Senegal, this protection did not appear to be as a result of high anti-AMA1 antibody titres, though they were prevalent in the population (Roussilhon et al. 2007). In yet another study carried out in Burkina Faso, total anti-AMA1 antibodies were not associated with protection from clinical disease yet anti-AMA1 IgG1 was associated with protection (RR 0.87, p=0.013) (Nebie et al. 2008). A recent systematic review supports the association between antibodies to AMA1 and protection from malaria (Fowkes et al. 2010).

1.5.2 Merozoite Surface Protein 2 (MSP2)

1.5.2.1 Location and structure of MSP2

MSP2 was discovered when a 45-55kDa protein was immune-precipitated by various monoclonal antibodies that had been shown to inhibit invasion of *P. falciparum* parasites (Stanley et al. 1985; Ramasamy 1987; Epping et al. 1988; Miettinen-Baumann et al. 1988; Smythe 1988). Using immune-fluorescent staining techniques and immuno-electronmicrosopy, MSP2 was shown to be one of the most abundant proteins on the surface of *P. falciparum* merozoites. It was also identified in low abundance in the ring stage (Smythe 1988) and in greater abundance on the surface of mature schizonts (Ramasamy 1987; Epping et al. 1988).

MSP2 is an integral-membrane protein that is anchored to the merozoite surface via a C-terminal GPI anchor (Smythe 1988; Gerold et al. 1996). It is an intrinsically unstructured protein in solution that forms amyloid-like fibrils under physiological pH (Low et al. 2007; Yang et al. 2007; Adda et al. 2009). The N-terminal end of the protein may also interact with the merozoite surface membrane leaving the central portion of the protein accessible to the external environment (Zhang et al. 2008).

1.5.2.2 Polymorphisms of MSP2

MSP2 contains numerous point mutations throughout its gene. The gene sequence is made up of conserved C- and N-terminal domains followed by flanking non-repeat variable sequences. The central domain contains the most variability and is made up of several repeat sequences. It is these repeat sequences that have been used to group the numerous MSP2 alleles into two main families (Thomas et al. 1990a; Fenton et al. 1991; Smythe et al. 1991). An analysis of seven different

MSP2 alleles split them into two families; one made up of 3D7, IC-1, IMR143, MAD71, NIG32; and the second made up of FC27 and K1 (Smythe et al. 1991). These two groupings are generally known as the '3D7-like' and 'FC27-like' MSP2 families. The 3D7-like family contains 4-8 amino acid repeats with five different amino acids being used whereas the FC27-like family has 12 and 32 amino acids repeated up to 4 times (Figure 1.6).

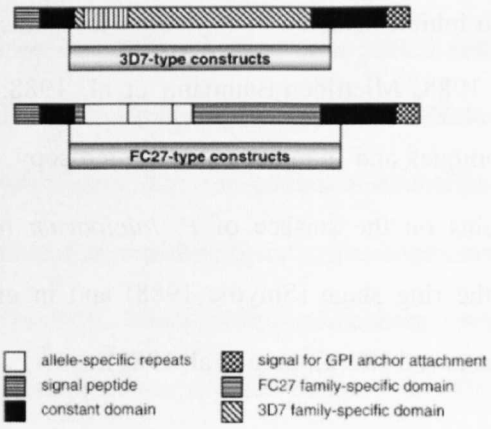

Figure 1.6 Schematic diagram of the structure of the two allelic families of MSP2 (Felger et al. 2003).

MSP2 appears to be under natural positive selection as shown by high synonymous/nonsynonymous rates at the MSP2 locus (Hughes and Hughes 1995). A larger sample of 100 MSP2 alleles from The Gambia, Tanzania, and Papua New Guinea were tested for balancing selection using the Ewens-Watterson test for neutrality and gave significant results for positive selection (Conway 1997). In yet another study, the evidence for positive selection at the MSP2 locus was not as convincing as in the above studies because the excess of non-synonymous substitutions was not very high (Escalante et al. 1998).

1.5.2.3 Function of MSP2

The precise function of MSP2 has not been identified but one was suggested from its initial identification using invasion inhibitory antibodies (Stanley et al. 1985; Ramasamy 1987; Epping et al. 1988; Miettinen-Baumann et al. 1988; Smythe 1988). Invasion inhibitory assays carried out

during initial MSP2 identification showed some inhibition which lends credence to a possible role for the protein in erythrocyte invasion (Epping et al. 1988; Miettinen-Baumann et al. 1988; Clark et al. 1989). This is supported by its increased expression during late schizogony and maximal expression in merozoites. However, a recent study found no invasion-inhibitory activity of MSP2 antibodies (Flueck et al. 2009). Amyloid-fibrils are present on the surface of bacteria and fungi and may have a role in the invasion of substrates. Their formation of a coat on the surface of these organisms has also been suggested as an immune evasion strategy (Gebbink et al. 2005). As MSP2 forms amyloid-like fibrils; this along with the invasion inhibition data may point towards a role in erythrocyte invasion.

That MSP2 was initially identified using previously defined invasion inhibitory monoclonal antibodies (13.4, 8G10/48, and 9E3/48) supports a role for it in invasion (Ramasamy 1987; Epping et al. 1988; Miettinen-Baumann et al. 1988; Clark et al. 1989). One study measured the effect of monoclonal antibodies 8G10/48 and 9E3/48 on actual parasite invasion of erythrocytes. 20% inhibition was observed at low mAb concentrations and increased in a dose-dependent manner to as high as 98%, thus proving that MSP2-specific invasion inhibition was possible (Epping et al. 1988). Recent observations have also shown that purified IgG from children with high antibody responses to MSP2 was more efficient at inhibiting parasite growth than that from children with low MSP2 responses (Courtin et al. 2009).

1.5.2.4 Vaccine potential of MSP2 in animal models

Though protection from parasite challenge was not seen in early animal vaccination studies, the immunization of *Saimiri* monkeys with an MSP2 allele from the 3D7-like family elicited antibody responses to both the 3D7-like and FC27-like families (Pye et al. 1991). MSP2 is not present in rodent malarias which has limited the ability to evaluate MSP2 as a vaccine. A later study where mice were immunised with octapeptides from the conserved N- and C-terminals from *P. falciparum* showed that the dominant antibody response was directed to variable regions of MSP2

and that the animals were protected against challenge with *P. chabaudi* (Saul et al. 1992); presumably the antibodies cross-reacted with related epitopes. Following on from the study by Saul et al; a chimeric protein made up of the N- and C- terminals of MSP2 was used to immunise mice. This immunization elicited an antibody response that was comparable in titres to that elicited by immunization with full-length recombinant MSP2 although the antibodies raised to the chimera did not recognize the same parasites as the those elicited by full-length MSP2 (Lawrence et al. 2000).

1.5.2.5 Naturally acquired antibodies to MSP2

Naturally-acquired antibodies to MSP2 can be detected in malaria-endemic populations. Total anti-MSP2 antibody prevalence is close to 100% by the age of ten in African populations (Taylor et al. 1998; Polley et al. 2006). Malaria transmission intensity affects the acquisition of these antibodies. In Kenya, antibody prevalence increased with age at a higher rate in a population where malaria transmission was higher (Polley et al. 2006). In an area with very low to negligible malaria transmission in Brazil, antibody prevalence to MSP2 did not rise higher than 30% (Scopel et al. 2007).

Antibody prevalence and titres increase with age, and titres tend to be higher when parasites are present in the host (Taylor et al. 1998; Metzger et al. 2003; Polley et al. 2006). Most of the antibody response to MSP2 appears to be to the polymorphic central part of the antigen. In two studies less than 5% of individuals recognized the conserved N- and C-terminal regions yet responses to the polymorphic regions could be detected in up to 95% of sera (Taylor et al. 1995; Metzger et al. 2003).

Naturally acquired allele specific antibodies to MSP2 have been detected. Both in malaria-endemic populations and in naive returned travellers suffering a primary infection, a higher proportion of antibodies to the circulating allelic family or infecting allelic family was detected (Tami et al. 2002; Felger et al. 2003). In Kenya, it was shown that higher antibody titres to one MSP2 allelic family

correlated with a higher parasite burden of the same allelic family whereas the antibody response to the other allelic family were significantly lower (Polley et al. 2006). These allele-specific antibodies are cross-reactive within the same family, with higher levels of cross-reactivity occurring in the more polymorphic 3D7-like family (Tonhosolo et al. 2001; Franks et al. 2003). Two studies have shown a lack of cross-reactivity between the two allelic MSP2 families, indicating that the acquired antibody response is towards the variable regions (Taylor et al. 1995; Franks et al. 2003). This is in agreement with the data obtained in animal models that was mentioned earlier.

The response to MSP2 consists predominantly of the cytophilic subclasses; IgG1 and IgG3. In several studies, IgG3 responses were of a higher prevalence and intensity in all age groups (Taylor et al. 1995; Taylor et al. 1998; Polley et al. 2006; Sarr et al. 2006; Tongren et al. 2006). Though IgG1 antibodies dominate in young children, the IgG3 response appears to begin overtake that of IgG1 by the age of two and continues to rise with increasing age until it is the dominant response (Taylor et al. 1998; Tongren et al. 2006). In a single study IgG1 antibody levels actually dropped with age (Taylor et al. 1998). In another study the antibody response of adult non-immune returned travellers experiencing a primary malaria episode displayed a predominantly IgG1 response (Eisen et al. 2007). These two results and those obtained by Tongren *et al* could indicate that IgG3 polarisation is due to cumulative malaria exposure, although a more recent study did not find any evidence of polarisation (Stanisic et al. 2009).

1.5.2.6 The role of anti-MSP2 antibodies in protection from clinical disease

The earliest immuno-epidemiological data that indicated a role for anti-MSP2 antibodies in protection from clinical malaria came from a study carried out in Papua New Guinea in preparation for a vaccine trial. High antibody levels to MSP2 were retrospectively associated with fewer episodes of fever and less anaemia (Al-Yaman et al. 1994). A year later, using a longitudinal study design it was shown that anti-MSP2 (3D7) antibodies were associated with a reduced risk of clinical malaria in the subsequent year (Al-Yaman et al. 1995). In Kenya, high antibody levels to

MSP2 were associated with protection from malaria over 28 weeks although low antibody levels were associated with an increased risk of malaria in the same period (Polley et al. 2006).

An interesting result regarding protection from clinical malaria was obtained in a study carried out in The Gambia on 3-8 year olds. Though the dominant IgG3 response to 3D7-like MSP2 alleles was associated with protection from clinical malaria over 6 months of follow-up, an IgG1 response to FC27-like MSP2 alleles was associated with an increased risk of malaria (Taylor et al. 1998). The authors suggest that the age-related switch from IgG1 to protective IgG3 anti-MSP2 antibodies may be an explanation. A later study in The Gambia confirmed that anti-MSP2 IgG3 antibodies to both the 3D7-like and FC27-like families were associated with a lower risk of clinical malaria (Metzger et al. 2003). A recent retrospective study in Senegal that followed-up individuals between the ages of 7 and 19 years for a year and associated antibody responses at the end of follow-up with protection from malaria found that IgG3 antibodies to MSP2(3D7) were associated with protection against malaria and high density parasitaemia whereas those to MSP2(FC27) were of borderline significance (Courtin et al. 2009). This is contrast to a recent meta-analysis that did not show a significant association between antibodies to MSP2 and protection from malaria (Fowkes et al. 2010).

1.6 Development of vaccines against malaria

An effective vaccine is likely to play a large role in the multi-faceted global fight against malaria and its debilitating mortality. Due to the complex life cycle of *P. falciparum*, the vaccine could act at various points with a number of possible outcomes. Key stages that the vaccine may target include the pre-erythrocytic, the asexual erythrocytic, or the sexual stages of *P. falciparum*. At these various stages the vaccine could prevent infection, reduce disease and/or parasite burden, or prevent transmission of the parasite.

1.6.1 Pre-erythrocytic stage vaccines

Pre-erythrocytic stage vaccines primarily target the sporozoite or infected hepatocyte and by preventing establishment of any infection will ideally result in sterile immunity. These vaccines will have no effect on any existing blood-stage infections. The best evidence of their potential came from the observations that irradiated sporozoites conferred complete protection from further challenge in animal models and humans (Nussenzweig et al. 1967; Hoffman et al. 2002). The logistics of providing sufficient numbers of irradiated sporozoites for use in the field are challenging so potential subunit vaccines based on key sporozoite antigens have been developed.

The most promising of these subunit vaccines is based on circumsporozoite protein (CSP) which is one of the most abundant proteins on the sporozoite surface. RTS,S/ASO2 is made up of the tandem repeat tetra-peptide and C-terminal T-cell epitopes regions of CSP fused to hepatitis B surface antigen S along with un-fused S antigen. The adjuvant ASO2 is an oil-in-water emulsion that contains immune-stimulants. Initial challenge trials in The Gambia gave up to 70% protection against parasitaemia over 9 weeks although this protection waned quite quickly (Bojang et al. 2001). In an extensive phase IIb trial carried out in Mozambique on 1142 children ages 1-4 years with 18 months follow-up, vaccination with RTS,S/ASO2A was able to elicit almost 50% protection from severe malaria and 30% protection from clinical malaria (Alonso et al. 2005). Another phase I/IIb trial of RTS,S/ASO2D in 214 infants in Mozambique with 6 months follow-up resulted in close to 70% efficacy against new infections (Aponte et al. 2007). A later clinical trial of RTS,S/AS02 on children below 5 years of age showed that this vaccine had an efficacy rate of 53% over 10 months of follow-up (Bejon et al. 2008). Although the efficacy of RTS,S/AS02 is well below that of vaccines used against other infections, these results make it the most promising malaria vaccine so far and phase IIb/III trials are underway in multiple African sites.

1.6.2 Erythrocytic stage vaccines

Erythrocytic stage vaccines target the antigens on the asexual parasite along with parasite-derived proteins inserted into the infected erythrocyte membrane. As the asexual stage is when the clinical symptoms of malaria occur, these vaccines aim to reduce the severity and possibly prevent disease by reducing or eliminating parasite loads. These vaccines are primarily targeted to children and pregnant mothers who bear the brunt of malaria morbidity and mortality. The major challenge that erythrocytic stage vaccines need to overcome is the substantial antigen polymorphism that exists in target antigens. Any potential vaccine will need to target those alleles that are most prevalent in the population and if possible, investigations should be carried out before any trial commences. The issue of antigen polymorphisms was highlighted in a study that was carried out to investigate prevalence of $MSP1_{19}$ haplotypes in a malaria-endemic population in Mali where a vaccine trial was about to start. The investigators found that out of 1363 malaria infections over 3 years, only 16% were of the vaccine haplotype (3D7) (Takala et al. 2007).

There are several malaria vaccines are based on a range of merozoite proteins, particularly MSP1, MSP2, MSP3, and AMA1 (Richards and Beeson 2009). Following disappointing results on an earlier MSP1 vaccine, two alternative vaccines MSP1-42(FVO)/Alhydrogel and MSP1-42(3D7)/Alhydrogel tested in naïve volunteers elicited a cross-reactive antibody response with very low levels of invasion inhibition (Malkin et al. 2007). For the first time, antibody-dependent cellular clearance of parasitaemia has be shown to be elicited by a vaccine based on conserved MSP3 regions and B cell epitopes (Druilhe et al. 2005). AMA1 and MSP2 vaccines will be discussed in detail below.

1.6.3 Transmission-blocking vaccines

Transmission blocking vaccines aim to reduce the rate of new infections in the vaccinated population which eventually leads to a reduction in overall disease. These vaccines will elicit herd immunity by interrupting the sexual cycle in mosquito vectors and prevent individuals from

passing on infections to others in the population. The first evidence of the possibility of blocking malaria transmission by vaccination came by the passive transfer of antibodies from chickens infected with *P. gallinaceum* that failed to kill parasites in their hosts yet killed parasites in mosquitos that were fed infected blood meals (Carter and Chen 1976; Gwadz 1976). The proteins involved in this immunity were localized to the gametocyte surface. P25 and P28 are ookinate antigens that are expressed by *P. falciparum* and *P. vivax* and were discovered by the use of transmission blocking monoclonal antibodies (Rener et al. 1983; Carter et al. 1984; Grotendorst et al. 1984). A single phase I trial in naïve volunteers vaccinated with a recombinant protein derived from the Pvs25 surface antigen of *P. vivax* ookinates has been reported (Malkin et al. 2005b). Vaccination with this antigen resulted in an antibody response that inhibited growth of ookinates in mosquitoes thus demonstrating effective transmission blocking activity.

1.6.4 AMA1 vaccines

There are currently a number of malaria vaccines based on AMA1 that are undergoing clinical trials. These are based on either the full length-AMA1 ectodomain sequence (AMA1-C1 and FMP2.1/AS02) or contain a peptide representative of the conserved region in the domain III loop (PEV301 and PEV3A). The vaccines PEV301 and PEV3A both contain a CSP repeat region peptide and utilize a virosome delivery system (Genton et al. 2007; Okitsu et al. 2007; Thompson et al. 2008). Both have tested safely during phase I/IIa trials in malaria-naïve volunteers and elicit a significant antigen-specific antibody response (Genton et al. 2007; Thompson et al. 2008). The potential for eliciting a long-lived affinity matured response over the course of immunisation was suggested by the two-fold increase in avidity of the detected antibodies (Okitsu et al. 2007). Though these results are encouraging, there remains a while until these formulations will be tested in malaria-endemic populations.

FMP2.1/AS02 is based on the AMA1 3D7 sequence formulated with the AS02 adjuvant. A phase I trial in malaria-naïve volunteers showed that this vaccine could elicit cellular and functional antibody responses as shown by inhibition of homologous parasite growth and inhibition of AMA1

processing (Polhemus et al. 2007). Due to the success of these results, a phase I trial was carried out in Mali on malaria-exposed adults (Thera et al. 2008). Anti-AMA1 antibodies increased over the course of immunisation and reached a 6-fold peak from baseline levels two weeks after the third vaccination. Significantly elevated levels of antibodies to AMA1 were measured at the end of the study (365 days post the first vaccination) and these antibodies were able to inhibit growth of both the homologous parasite (3D7) along with a heterologous strain (FVO) (Thera et al. 2008). Vaccination of malaria naive adults with AMA1/AS02A followed by sporozoite challenge resulted in a significant reduction in parasite growth rates as detected by Q-PCR, but not light microscopy (Spring et al. 2009). Phase I and II trials in children are currently underway. At the time of writing, results of a recent phase 2 trial in Mali were presented at the MIM Pan-African malaria conference, 2009. The AMA1/AS02A containing AMA1-3D7 gave significant protection against malaria episodes caused by 3D7-like alleles.

Of the malaria vaccines based on AMA1; AMA1-CI has progressed furthest in clinical trials. AMA-CI contains two AMA1 variants that are based on the full ectodomain sequence of 3D7 and FVO that are adsorbed on Alhydrogel. These variants differ at 25 amino acid position and were picked in order to best represent the wide repertoire of AMA1 variants that are present in malaria endemic regions (Malkin et al. 2005a). Phase I trials in naïve volunteers gave promising antibody responses to both the variants after the second and third immunization, and purified IgG from the vaccines was able to inhibit growth of 3D7 and FVO parasites (Malkin et al. 2005a). AMA1-CI was the first AMA1 vaccine to be tested in a malaria-endemic population. Antibody levels in Malian adults increased rapidly after the first two vaccinations in a dose-dependent manner but began to drop two weeks after the second vaccination, and did not rise after a third vaccination (Dicko et al. 2007). There was also no increase in the growth inhibitory activity of the vaccine induced antibodies. In order to reconcile this non-inhibitory response with the inhibitory one seen in malaria-naïve vaccinees, a study comparing vaccine-induced and naturally acquired anti-AMA1 antibodies was carried out (Miura et al. 2008). This study revealed that the antibodies elicited by vaccination were as effective as naturally acquired ones at inhibiting parasite growth when they were affinity purified from serum, but part of the natural antibody response to AMA1 or other

antigens included a fraction of antibodies that blocked the action of AMA1-specific invasion inhibitory antibodies. In a phase I trial in children from the same area, AMA1-C1 elicited an antibody response two weeks after the first vaccination that peaked at day 42 and fell by day 100 (Dicko et al. 2008). Only two doses of AMA1-C1 were administered in this trial which may explain the short-lived response. The authors suggested that as children have had less cumulative exposure to malaria, they may require three doses of the vaccine to elicit longer-lived antibody responses. In a Phase I trial in children there was no protection against high density parasitaemia or clinical malaria conferred by this vaccine (Sagara et al. 2009). Recent evaluation of the immune response of non-exposed AMA1-C1 vacinees indicated that this vaccine may also be able to induce the development of memory T-cells (Huaman et al. 2009).

1.6.5 MSP2 Vaccines

Initial studies in development of a malaria vaccine based on MSP2 were not encouraging. The first test of a combination vaccine containing MSP2 and CSP constructs in naïve volunteers failed to protect against parasite challenge although antibodies to both MSP2 and CSP had been elicited by vaccination (Sturchler et al. 1995). Another phase I trial on naïve volunteers using peptide fragments of MSP1, MSP2, RESA, and a CSP T-cell epitope with Montanide ISA720 adjuvant elicited weak antibody responses to all three antigens along with a strong T-cell response (Saul et al. 1999). The authors suggest that this result could have been due to an ineffective adjuvant reaction or the immunogenicity of the peptide constructs used.

To date, Combination B which contains fragments of MSP1 (K1), MSP2 (3D7), and RESA with Montanide ISA720 adjuvant is the only MSP2-containing vaccine to go through clinical trials in a malaria-endemic area. After successful tests for safety and immunogenicity in Papua New Guinean adults, a phase I/IIb trial in 120 5-9 year olds in the same area was carried out (Genton et al. 2000; Genton et al. 2002; Genton et al. 2003). Combination B was safe in children and managed to elicit measurable humoral and cellular immune responses (Genton et al. 2003). Combination B proved

efficacious with a 62% reduction in parasite load but did not protect against clinical disease in one year of follow-up (Genton et al. 2002). Half the children in the trial were pre-treated with SP to clear parasitaemia before administration of the vaccine. This treatment may have interfered in the action of the vaccine as those children who underwent treatment did not clear their parasitaemia and had significantly lower cellular responses to MSP1 (Genton et al. 2003). In fact, there was an increase in the incidence of disease in vaccinated children in the subsequent year, most of which was caused by non-vaccine MSP2 FC27 parasites (Genton et al. 2002). Combination B appeared to elicit a strain-specific response to MSP2 that was confirmed by the low antibody titres to MSP2 FC27 in vaccinated children (Fluck et al. 2004). A new formulation of Combination B that contains MSP2 antigens from the 3D7-like and FC27-like family is currently undergoing clinical trials.

1.7 Longitudinal studies of antibodies to asexual antigens of *P. falciparum*

There are only a few longitudinal studies with repeated antibody measurements to investigate the acquisition of various antibodies to malarial antigens in endemic populations that have been carried out in children (Branch et al. 1998; Giha et al. 1999; Udhayakumar et al. 2001; Biswas et al. 2008). To date only two studies have shown a stable persistence of antibody levels to MSP1 and AMA1 in adults (Riley et al. 1993; Udhayakumar et al. 2001). Most of the studies showed that antibodies to *Pf*MSP1, *Pv*MSP1, *Pf*AMA1, *Pv*AMA1 and *Pf*EMP1 are short-lived and decline significantly over the study period though antibodies to *Pv*AMA1 could be detected in a few individuals up to 7 years after an outbreak of *P. vivax* malaria in Brazil (Branch et al. 1998; Cavanagh et al. 1998; Giha et al. 1999; Soares et al. 1999a; Morais et al. 2006; Biswas et al. 2008). Antibody responses were associated with parasitaemia at the time of sampling or an episode prior to sampling time. All the mentioned studies have been limited in sample size and length of follow-up and have not examined the relationship between antibody responses and malaria episodes in detail. A single study in

infants followed up from birth to the age of one year found that anti-MSP1 antibodies were protective against subsequent parasitaemia and febrile illness (Branch et al. 1998).

Longitudinal studies allow the individual development of dynamic outcome variables to be studied over time. The power of longitudinal studies lies in the ability to distinguish changes over time within individuals from the differences in a cohort of individuals at baseline. The variability that is inherent across individuals due to possible unmeasured variables that persist in time will be minimized as each individual serves as their own control (Diggle et al. 2002).

As antibodies to merozoite antigens are generally short-lived, the calculation of a protective response from a single time-point may not give a true picture of the contribution of antibodies to long-term immunity or an individual's immune status. Longitudinal analysis of antibody levels during the most intensive period of acquisition of immunity in malaria endemic areas (childhood) may give a more detailed picture of antibody-associated immunity. Measurement of antibody levels at several time points is required to properly assess their protective abilities. The data that is collected in longitudinal studies is likely to give the best picture of acquisition of immunity to malaria and the dynamic role that different factors play in protection against disease.

1.8 Rationale of this study

This project aims to investigate the acquisition and maintenance of antibodies to *P. falciparum* AMA1 and MSP2 in childhood and examine their role in protective immunity to malaria using samples from well defined cohort and longitudinal studies in a malaria-endemic part of Kenya. As stated earlier` natural immunity to malaria is non-sterile, protects against clinical disease, and appears to be complete in adults living in malaria-endemic areas. An extension of this is that patent parasitaemia along with clinical disease (mild or severe) could be markers of susceptibility to malaria in individuals living in malaria-endemic areas. Those individuals without measureable parasitaemia or symptoms in spite of continued exposure may be considered as having attained effective anti-parasite and clinical immunity to malaria. To date there are no definitive empirical

markers of this immune state. There is no measurement that can be taken in an individual and used to consistently predict the likelihood of disease in a subsequent time period. To this end various markers of an active immune response (antibodies, cytokines, and activated cells) have been used to attempt to define the immune status of individuals exposed to malaria.

The most common of these immune markers is the presence or absence and levels of antibodies to *P. falciparum* in sera. The earliest empirical evidence of a natural antibody response playing a role in immunity to malaria came from experiments where the transfer of purified immunoglobulin from immune adults to children suffering from clinical malaria led to the successful clearance of parasitaemia in 8 out of 12 children (Cohen et al. 1961). This result was confirmed in other studies and has led to the prolific measurement of antibodies in animal and human studies as markers of immunity and/or exposure to malaria (Sabchareon et al. 1991; Hogh et al. 1995; Branch et al. 1998). As already discussed, antibodies exert their effects by inhibition of invasion of merozoites into erythrocytes, inhibition of adhesion of parasitised erythrocytes to endothelial walls, opsonic phagocytosis of infected erythrocytes, and antibody-dependent immune cell mediated inhibition or killing of infected cells.

Immuno-epidemiological studies where antibodies to various antigens have been measured in malaria-endemic populations have so far given us great insight into development and maintenance of the immune response to malaria. The majority of these studies have measured antibodies at one time point and associated them with protection or susceptibility to malaria in the future. Implicit in this method is the assumption that the ability of an individual to mount an immune response can be assessed at a single time point. Simple observations of individuals living in malaria endemic areas show that this is not the case. Immunity to malaria is a dynamic process that takes place over a number of years in different stages, is dependent on the age of the individual, the level of malaria transmission in the area, and is both strain specific and cross-reactive (Bray et al. 1962; Collins et al. 1966; Jeffery 1966; Marsh and Howard 1986; Trape et al. 1987; Forsyth et al. 1989; Baird et al.

1991; Baird et al. 1993; Snow et al. 1997; Marsh and Snow 1999; Hudson Keenihan et al. 2003; Keenihan et al. 2003; Marsh and Kinyanjui 2006).

Numerous immuno-epidemiological studies measuring the antibody response to these two merozoite proteins and investigating their role in protection have been carried out. The results from these studies are not as clear as those from animal models and conflict with each other. So far three studies provide evidence that antibodies to AMA1 are associated with protection from malaria for up to 8 months after sample time (Polley et al. 2004; Gray et al. 2007; Osier et al. 2008), although in one study this was only in combination with an antibody response to MSP3 (Osier et al. 2008). Three studies have given the opposite result, with antibodies to AMA1 not being associated with protection from clinical malaria (John et al. 2005; Roussilhon et al. 2007; Nebie et al. 2008) Four studies link antibodies to MSP2 with protection from future parasitaemia and clinical malaria (Al-Yaman et al. 1994; Al-Yaman et al. 1995; Metzger et al. 2003; Polley et al. 2006), and another implies that the protective effect is dependent on an antibody response to both MSP2 and MSP3 (Osier et al. 2008). One study showed that antibodies to FC27-like MSP2 alleles were associated with increased risk of malaria in The Gambia (Taylor et al. 1998). Overall, data from malaria-endemic areas show that antibodies to MSP2 were not significantly associated with protection from clinical malaria whereas there was better evidence for those to AMA1 (Fowkes et al. 2010). The relative paucity of immune-epidemiological data from malaria-endemic areas when these antigens are already in Phase I/II trials as potential malaria vaccines is not ideal. The fact that malaria appears to be undergoing an epidemiological transition in those areas that the vaccines are being targeted underscores the lack of substantial immuno-epidemiological data that may guide future immunisation strategies.

There have been few longitudinal studies using repeated antibody measurements over time investigating the acquisition of antibodies to malarial antigens during childhood (Branch et al. 1998; Cavanagh et al. 1998; Giha et al. 1999; Udhayakumar et al. 2001; Morais et al. 2006; Kinyanjui et al. 2007). Most studies have been limited in sample size and length of follow-up and have not examined the relationship between malaria episodes in detail. Some studies suggest that

antibodies elicited by exposure to *P. falciparum* and *P. vivax* may have a very short half-life (Branch et al. 1998; Cavanagh et al. 1998; Soares et al. 1999a; Kinyanjui et al. 2007). Measurement of antibody levels at several time points is required to properly assess their protective abilities. The data that is collected in longitudinal studies gives the best picture of acquisition of immunity and the dynamic role that different factors play in protection against disease.

The studies presented in this thesis will add to the current body of longitudinal immunological data on the acquisition of malaria during the period (childhood) when immunity is being acquired in malaria endemic populations. As this study is being carried out in a mid-low malaria transmission area with a general reduction in malaria due to various human activity and climatic factors, this study will sketch part of the currently emerging picture regarding acquisition of long-term immunity against malaria at a time when severe malaria incidence is falling and could have a great impact on vaccination development and immunisation strategy.

1.9 Aims of this study

There are four main objectives of this study:

1. Examine the dynamics of antibody acquisition and maintenance of antibodies to merozoite antigens over time in relation to age, exposure, active parasitaemia, and erythrocyte polymorphisms.
2. Determine whether antibodies to AMA1 and MSP2 are indicators of protection from clinical malaria or markers of exposure.
3. Examine the acquisition of antibodies to a functional epitope of AMA1 and the relationship with protection from clinical malaria.
4. Examine the effect of repeated exposure on antibody repertoire and IgG isotypes specific to AMA1.

The above objectives of this study will be fulfilled by investigating the following hypotheses:

1.9.1 Hypothesis 1: Antibody levels fluctuate over time and become more stable with increasing age and exposure

Longitudinal studies show that the antibody response to various malaria antigens is short lived and fluctuant in young children (Branch et al. 1998; Kinyanjui et al. 2007) but remains stable in adults (Riley et al. 1993; Taylor et al. 1996; Udhayakumar et al. 2001). These studies were carried out in areas of high transmission or in the case of Kinyanjui *et al*, investigated antibody levels in children convalescing from malaria that required hospitalisation. Several studies also show that antibody levels increase with increasing malaria transmission (Trape 1987; Snow et al. 1997; Marsh and Snow 1999). Most of the data available has been collected in areas of saturating exposure so the separate effects of age and exposure have been difficult to tease apart. What is still unknown is the direct effect of measured episodes of exposure on antibody levels during childhood. I intend to investigate this by examining the relationship between antibody levels, age, and parasitemic episodes using both cross-sectional and longitudinal data.

1.9.2 Hypothesis 2: Maintenance of antibodies requires ongoing exposure to *P. falciparum*

Antibody levels are seen to increase with increasing malaria endemicity and tend to mirror malaria transmission rates in areas of seasonal transmission (Ramasamy et al. 1994; Jakobsen et al. 1997; John et al. 2002). In longitudinal studies antibody levels to different antigens have been associated with parasitaemia at the time of sampling or an episode prior to sampling time (Branch et al. 1998; Cavanagh et al. 1998; Giha et al. 1999; Soares et al. 1999a; Morais et al. 2006; Biswas et al. 2008). It is not well understood whether individuals are able to maintain their high antibody response or to acquire a significant antibody response in the face of reductions in malaria transmission. I intend to look at this by investigating the association between different levels of exposure and maintenance of antibody responses over the study period.

1.9.3 Hypothesis 3: Acquisition of antibodies varies between different merozoite antigens and different polymorphic variants of the same antigen

Antibody responses to different antigens can be both strain specific and cross-reactive. Field studies have shown that antibodies to different variants of the same antigen tend to be correlated but antibodies to different antigens may not be correlated (Bray et al. 1962; Collins et al. 1966; Jeffery 1966; Forsyth et al. 1989; Marsh et al. 1989). Though many studies have studied the acquisition of antibodies in childhood by looking at the antibody prevalence and levels in cross-sections of malaria-endemic populations, substantial antibody data from the same children over a number of years is not available for the comparison of differential acquisition. I intend to compare the acquisition of antibodies to different recombinant AMA1 (3D7, HB3, and W2mef) and MSP2 (3D7 and FC27) variants.

1.9.4 Hypothesis 4: Maintenance of high levels of antibodies to AMA1 and MSP2 are associated with protection from clinical malaria

In prospective studies with follow-up for the incidence of clinical malaria, antibodies to both AMA1 and MSP2 have been associated with protection from malaria in some instances (Al-Yaman et al. 1994; Al-Yaman et al. 1995; Polley et al. 2004; Polley et al. 2006; Gray et al. 2007; Osier et al. 2008). Yet some longitudinal studies show that antibody responses are associated with parasitaemia at the time of sampling or an episode prior to sampling time. What is unknown is whether maintenance of higher antibody levels will protect against malaria or whether the high levels are indicative of prolonged or repeated exposure. I intend to investigate this by analyzing the risk of clinical malaria in individuals with different antibody responses to AMA1 and MSP2, and taking account of the factors influencing the induction and boosting of antibodies.

1.9.5 Hypothesis 5: Antibodies to a defined invasion-inhibitory epitope on AMA1 are naturally acquired in malaria endemic populations and contribute to protection from clinical disease

Naturally acquired antibodies to AMA1 are able to inhibit invasion of erythrocytes by merozoites *in vitro*, but little is known about the prevalence and extent of these responses (Hodder et al. 2001; Healer et al. 2004). Several monoclonal antibodies to AMA1 can also inhibit invasion *in vitro* (Coley et al. 2001; Coley et al. 2006; Collins et al. 2007; Collins et al. 2009). Antibodies which bind to the same epitope as one of these inhibitory mAb have also been detected in malaria exposed individuals (Coley et al. 2007). As this is a functional inhibitory epitope on AMA1, it is likely that presence of these epitope-specific antibodies is associated with protection from clinical malaria. I intend to investigate the acquisition of mAb 1F9 epitope-specific antibodies and determine their potential contribution to protection against clinical malaria

1.9.6 Hypothesis 6: Erythrocyte polymorphisms affect the acquisition and maintenance of antibodies

Both the sickle cell trait (HbAS) and α+ thalassemia confer considerable resistance to severe and complicated malaria (Williams et al. 2005b; Williams et al. 2005d). The immunity conferred by HbAS is in part due to the physical nature of the erythrocytes as well as immune regulation (Marsh et al. 1989; Bayoumi et al. 1990; Abu-Zeid et al. 1992). The protective action of α+ thalassemia could be via inhibition of parasite growth/invasion or other immunological mechanisms (Williams et al. 2005d; Wambua et al. 2006; Fowkes et al. 2008). Although associations between haemoglobinopathies and antibodies have been reported in cross-sectional studies, to date there are no studies on the acquisition and maintenance of antibodies to malaria antigens in HbAS or α+ thalassemic individuals. I intend to investigate the acquisition and maintenance of antibodies in these individuals and compare them with normal individuals.

1.9.7 Hypothesis 7: The induction, maintenance, and protective effects of isotype and subclass antibody responses to AMA1 is dependent on amount of *Plasmodium* exposure and age

Studies in Africa and Asia have shown that the cytophilic IgG1 and IgG3 subclasses are the predominant protective antibody responses to merozoite antigens whereas IgM predominates in younger children who are susceptible to disease (Bouharoun-Tayoun and Druilhe 1992; Taylor et al. 1995; Shi et al. 1996; Rzepczyk et al. 1997; Polley et al. 2006; Nebie et al. 2008; Stanisic et al. 2009). A number of factors including host genetics, antigen type, cumulative exposure, and age have been implicated in determining the predominant subclass response to merozoite antigens (Nguer et al. 1997; Taylor et al. 1998; Aucan et al. 2000; Aucan et al. 2001; Garraud et al. 2002; Ntoumi et al. 2002; Ntoumi et al. 2005; Scopel et al. 2005). Increasing age has also been suggested to polarize the antibody response to IgG1 for AMA1 though this was not seen in a more recent study (Scopel et al. 2006; Tongren et al. 2006; Stanisic et al. 2009). Anti-AMA1 antibodies in malaria endemic populations are predominantly IgG1 and IgG3 with very little IgG2 or IgG4 detected (Riley et al. 2000; Polley et al. 2004; Metenou et al. 2007; Nebie et al. 2008; Stanisic et al. 2009). I will investigate the patterns of subclass (IgG1, IgG3) and isotype (IgG, IgM) antibody responses to AMA1(3D7) and compare the effects of various factors – in particular repeated exposure and increasing age – on the magnitude of the antibody response and assess whether specific isotype or subclass responses are associated with protection against clinical malaria in this cohort of children.

2 Materials and Methods

2.1 Study Area and Population

Kilifi District covers an area of 4,779 km^2, borders the Indian Ocean, and is located in the Coast Province of the Republic of Kenya. It is one of the poorest districts in the country and has a population of 650, 000. This study was carried out using samples from two locations 20km apart within Kilifi district with similar demographic and socioeconomic characteristics (Mwangi et al. 2005). Kilifi district is predominantly settled by people of the Mijikenda tribe with farming as its main income generation activity.

Most of the malaria in the study area results from *P. falciparum* infection which is predominatly transmitted by *An. gambiae s.l* (Mbogo et al. 1993; Mbogo et al. 2003; Mwangi et al. 2003). Transmission occurs biannually, during the rainy seasons of May-July and November-December. Ngerenya is located north of Kilifi creek and is within an area that has been under demographic surveillance since 1991 (Snow et al. 1993; Nevill et al. 1996). Chonyi is south of Kilifi creek and is characterised by more fertile soils and a hillier landscape. Negrenya is a low transmission area with an entomologic inoculation rate (EIR) of 10 infective bites per year whereas Chonyi is considered a mid-high transmission setting with an EIR of 22-53 infective bites per year (Mbogo et al. 1995; Mbogo et al. 2003).

2.2 Study Design

This study comprises of two components nested within an ongoing longitudinal study that was established in 1998 to investigate the epidemiology of non-severe malaria in Kilifi district.

1. The major part of this study is an observational longitudinal cohort study with 6 sampling points approximately six months apart. The cross sectional bleeds were carried out in May

2002, October 2002, May 2003, October 2003, May 2004, and October 2004 with continuous active weekly case detection.

2. A smaller cross sectional cohort study made up of two cohorts within the larger non-severe malaria study.

The non-severe malaria study was established in 1998 in Ngerenya included both adults and children and continued for a few months before recruitment in Chonyi began. 819 participants were enrolled in Ngerenya and 783 participants in Chonyi (Mwangi et al. 2005). Verbal consent in the local language was sought from all adults and from parents or primary care givers of all children less than 14 years of age. Consent was also sought from the heads of households and village elders. Written consent (thumb-print or signature) was then obtained for participation in the study with the understanding that they could withdraw from the study at any point. Active weekly surveillance of non-severe malaria was undertaken from enrolment. The study was modified in August 2001 to include 300 children under the age of 8 years in Ngerenya alone. Active weekly surveillance continued in the original manner. Children born into study households in Ngerenya were recruited into the study at birth. Participants exited the study if consent was withdrawn, if they moved out of the area for more than 2 months, or if they died. All participants exited the study by the age of 10 years.

2.3 Sample collection

Cross sectional sampling was carried out in Ngerenya only in September 1998. Further cross sectional bleeds occurred in both Chonyi and Ngerenya in July 1999, March 2000, July 2000, October 2000, March 2001, and June 2001 (children and adults) (Mwangi 2003; Mwangi et al. 2005). The surveys were taken during 'high' (June, July) and 'low' (March, October) malaria transmission seasons which correspond to the rainy and dry seasons. Axillary temperatures were measured and blood smears taken for all participants and serum collected for further studies.

On modification of the study in August 2001, cross sectional sampling was initiated just before the malaria seasons in May and October (designated 'May' and 'October' bleeds). 5ml of venous

blood was collected from each participant along with a blood smear and anthropometric data. The cross sectional bleeds took place over a few weeks due to the distances between participating households and other logistical issues. Sampling periods are as follows:

- May 2002 bleed; 13 May to 28 May 2002
- October 2002 bleed; 11 October to 27 November 2002
- May 2003 bleed; 12 May to 23 May 2003
- October 2003 bleed; 21 October to 9 February 2004
- May 2004 bleed; 26 April to 16 June 2004
- October 2004 bleed; 18 October to 9 December 2004

Parasitaemia was measured by light microscopy and one hundred high power fields were examined before a slide was considered negative. Parasites were counted per 200 white blood cells (WBCs) and density was calculated using an average count of 8000 WBC/μl of blood. Samples were taken to the KEMRI CGMR-C laboratories where they were processed and the separated serum stored at -20°C for use in antibody studies. Enrolled individuals were assigned ID numbers that included the prefix 'N' (Ngerenya) or 'C' (Chonyi) to identify their area of residence. Serum used for the study was obtained from labelled serum separator tubes and was diluted to 1:25 in phosphate buffered saline (PBS) with 0.001% sodium azide for preservation. Samples could then be stored at 4°C but were kept at -20°C or -80°C for long term storage.

2.4 Weekly Surveillance

All households with individuals recruited into the study were visited by a fieldworker once a week. Each participant had their axillary temperature measured by a digital thermometer (Becton Dickinson). A temperature greater than 36°C was confirmed by three measurements. Participants with a fever (temperature $\geq$ 37.5°C) or a history of fever had a blood smear performed and if unable to make it to the study clinic, the field workers dispensed malaria treatment. Sulfadioxine-pryimethamine (SP) was the first line treatment, in accordance with national guidelines.

2.5 Case Definition

As the clinical signs of malaria are nonspecific, the presence of parasites accompanied by fever may not be enough to define a clinical episode of malaria in an endemic area. Clinical mild malaria is defined as any parasitaemia accompanied by a measurable fever in children <1 year and a parasitaemia ≥2500/μl of blood accompanied by fever in children ≥1 year (Mwangi et al. 2005). Any incidence of a parasite positive slide in the intervals between sampling is referred to as a parasitemic episode (whether the child was symptomatic or asymptomatic). Any mild malaria episodes as defined above are therefore a subgroup of parasitemic episodes.

2.6 Cohort study - Study populations, sample collection, and surveillance

Samples were obtained from cohort studies based in Kilifi District. The study samples comprised of 130 serum samples from Ngrerenya collected in September 1998 (ages 0-85 years). 276 samples from Chonyi were collected in October 2000 from individuals 0-55 years of age. All individuals were monitored weekly by active and passive surveillance for symptomatic illness and malaria; any febrile episodes were investigated for malaria. Serum from 20 non-exposed British and Australian donors were used as reference controls.

2.7 Recombinant *P. falciparum* antigens

2.7.1 AMA1

Recombinant, refolded AMA1 (3D7, HB3, W2mef) ectodomain was received from Anders RF. Full details of preparation can be found elsewhere (Hodder et al. 2001). Briefly, the *P. falciparum* ectodomain was expressed in *E. coli* with an N-terminal hexa-His tag. Nucleotide sequences were amplified from genomic DNA using *Pfu* DNA polymerase and oligonucleotide primers. The amplified products were digested with *Bam*HI and *Pst*I, and ligated into pDs%6/RBs11/6xHis, and transformed into *E. coli* strain JPA101. The protein was refolded using previously established methods by diluting in a buffer containing reduced and oxidised glutathione (Anders et al. 1998).

2.7.2 MSP2

Recombinant MSP2 (3D7 and FC27) was also received from Anders RF. Full length MSP2 was expressed in *E. coli* with C-terminal His_6 tags and then purified using nickel chelate anion-exchange and reversed-phase chromatography.

2.8 Other Antigens

2.8.1 Schizont Extract

Crude schizont antigen was obtained from Mosobo M. and Lowe B. at the KEMRI-Wellcome Trust Research Programme, Kilifi. The antigen was prepared from the *P. falciparum* A4 clonal line, derived from the ITO parent strain, by sonicating highly synchronised schizont stage parasites on ice. Uninfected erythrocytes sonicated at the same frequency were used as a control antigen. The antigens were stored at -80°C and thawed on ice just before use.

2.8.2 Tetanus Toxoid

Vaccine grade tetanus toxoid was obtained from Commonwealth Serum Laboratories (CSL), Australia.

2.9 Monoclonal Antibodies

Monoclonal antibodies (mAb) were obtained from Coley AM and Anders RF. They were generated using standard methods (Harlow and Lane 1988). MAb 1F9, 2C5, and 5G8 were generated by immunization of mice with recombinant refolded 3D7 AMA1. MAbs were produced in hybridoma cell cultures and purified by protein G chromatography (Coley et al. 2007). The reactivity of the MAb to AMA1 was assayed by ELISA, and immunofluorescence of 3D7 schizonts and merozoites (Coley et al. 2001).

2.10 Enzyme Linked immunosorbent Assay

Enzyme linked immunosorbent assay (ELISA) is the standard *in vitro* method for measuring antibody titres in plasma. Three different methods were used all based on standard ELISA techniques and are described below.

2.10.1 Standard ELISA

Meausrement of the total anti-AMA1 IgG titres was carried out using standard ELISA techniques. Dynex Immunolon 4HBX ELISA plates (Dynex Technologies Inc) were coated with 100µl of 1µg/ml in PBS of antigen (3D7 AMA-1) per well and left overnight at 4°C. Wells were washed three times using a wash buffer (PBS-Tween 20, 0.05%). 100µl/well of blocking buffer (PBS-Tween 20, 10% skimmed milk powder) was added to each well and the plates incubated at 37 °C for two hours. After a single wash with wash buffer, 100µl/well of a 1:2000 dilution of sera in sample buffer (PBS-Tween 20, 5% skimmed milk powder) was added to each well in duplicate and incubated at room temperature for one hour. The plates were washed three times and incubated with 100µl/well of secondary antibody (HRP conjugated polyclonal rabbit anti-human IgG, Dako) diluted at 1:2500 in sample buffer for one hour at room temperature. After three washes with wash buffer, 100µl/well of substrate solution (per 96 well plate: 2.45ml 0.1M Citric Acid, 5.2ml 0.1M Na_2HPO_4, 2.35ml H_2O, 4mg *o*-Phenylenediamine dihydrochloride [OPD], activated with 4µl H_2O_2 just before use). The plates were then covered and incubated at room temperature for 10-15 minutes. The reaction was stopped by addition of 20µl/well 2M sulphuric acid and absorbance measured at 492nm.

Standard ELISA was also used for measurement of the total antibody response to blood-stage antigens, as a marker of exposure. A4 schizont extract produced using established methods was used as the antigen at a dilution of 1:1000 (Ndungu et al. 2002). Uninfected sonicated erythrocytes were used as control antigen. The standard ELISA protocol above was used with slight changes in sera dilution (1:1000) and secondary antibody dilution (1:2000). Measurement of anti-IgM antibodies to recombinant AMA1 was carried out using the standard ELISA technique at a 1:100

serum dilution. IgM antibodies were detected using HRP conjugated goat anti-human IgM (Chemicon, AP114P) at a 1:2500 dilution.

Any ELISA sample with a difference greater than 25% between duplicates was repeated. Any plate with more than a quarter of the samples having a discrepancy of greater than 25% between duplicates was also repeated. A mean value for each sample was calculated and then all results were standardized using the positive controls on each plate. The background absorbance from negative control wells (using sera from a panel of non-exposed individuals) was then subtracted from each value.

2.10.2 High Throughput ELISA

High throughput screening of potential drugs using robotics is an expanding practice in the pharmaceutical and biotech industries and facilitates the testing of very large compound libraries. Various methods are employed with certain factors in common. The standard ELISA technique described above was adapted for use with these high throughput systems in order to reduce the volume of sera used and increase the number of samples assayed in a single experiment and thereby facilitate testing for multiple antibodies using all samples from the main Ngerenya cohort described below.

To measure antibodies to multiple antigens a high throughput ELISA system was developed. This system uses the Multi-Track (Perkin Elmer) and Multi-Position Dispenser (Perkin Elmer) and allows antibody measurement of up to 10,000 samples in a single assay run. IgG to AMA1 (3D7, HB3, and W2mef variants), MSP2 (3D7 and FC27 variants), and Tetanus Toxoid were measured in all samples from the Ngerenya study. Specific isotypes (IgG1, IgG3, and IgM) were also measured in all samples for AMA1 (3D7).

Nunc Maxisorp 384 well plates (Nunc) were coated with the antigen of interest by addition of 50µl per well of antigen diluted in PBS using a Multidrop 384 (Thermo Labsystems). AMA1 (3D7, HB3, W2mef) and MSP2 (3D7, FC27) were coated at a concentration of 0.5µg/ml, and Tetanus

Toxoid at a concentration of 0.4LFU/ml. The plates were sealed and incubated overnight at 4°C. Plates were then washed twice using PBS with 0.25% Tween 20 (wash buffer). Non-specific binding was blocked by addition of 100µl per well of PBS with 0.1% Casein (Pierce) and 0.05% Tween 20. The plates were then sealed and incubated overnight at 4°C. The plates were washed once before addition of sera. 50µl of sera diluted in PBS with 0.1% Casein and 0.05% Tween 20 was added to each well and incubated for 2 hours at room temperature. Total IgG was measured at a sera concentration of 1:4000 (AMA1), 1:1000 (MSP2), and 1:500 (Tetanus Toxoid). IgG1, IgG3, and IgM to AMA1 were measured at a sera concentration of 1:100.

Inter-well variability was examined by comparing results from duplicate wells in the same assay. 384 samples from the Ngerenya cohort were tested in a single assay in duplicate. The correlation between duplicates was extremely high (correlation coefficient = 0.96) with no outliers (Figure 2.1). This strong association assured us that it would be feasible to carry out further testing of samples in single wells.

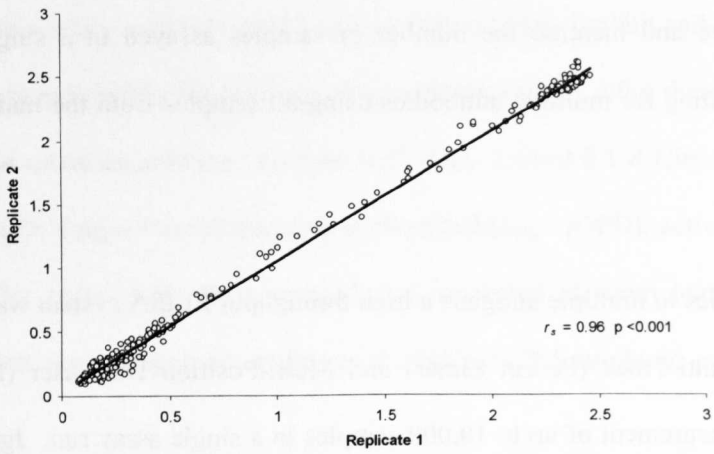

Figure 2.1 Correlation between duplicate samples in the same assay, high throughput ELISA. Spearman rank correlation, $r_s = 0.96$: $p < 0.001$; n = 384.

After the sera incubation, the plates were washed five times using wash buffer. 50µl of HRP conjugated anti-human antibody diluted at 1:2500 in PBS with 0.1% Casein and 0.05% Tween 20 was added to each well and the plates were incubated for 1 hour at room temperature. HRP

conjugated rabbit anti-human IgG (AP316P, Millipore Australia) was used in the assays measuring total IgG.

The plates were then washed five times in wash buffer and 50µl of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (A3219, Sigma) was added to each well. The plates were incubated for 30 minutes in the dark to allow for sufficient colour change. Addition of 50µl of 1% SDS per well stopped the reaction and absorbance was read at 405nm using an Envision plate reader (Perkin Elmer). Antibody levels were expressed as an absorbance value (OD).

A pool of sera from Papua New Guinean adults and sera from individual adults were used as positive controls and included on each 384 well plate. These were used to standardize the absorbance readings across all the plates for each antigen. Sera from non-exposed Australian individuals were used as negative controls. The reproducibility of the assay was evaluated by comparing absorbance values obtained from two independent experiments carried out on different days and using the same batch of samples. A coefficient of variation (CV) of 1.2% was observed for this comparison which indicated good reproducibility. The results obtained from the optimized high-throughput ELISAs were compared to those obtained using a standard ELISA method. The same reagents and samples were used in both assays. Samples were tested in duplicate in the standard ELISA and a mean value obtained, compared with a single well in the high-throughput ELISA. 272 samples from the May 2003 cross-sectional bleed were tested. There was a strong correlation between both methods (r = 0.90) although there were a few non-concordant pairs (Figure 2.2). Plate-to-plate reproducibility was good with positive controls that were tested on each plate strongly correlating ($r > 0.90, p < 0.001$).

Detection of IgM was done using 50µl of a 1:2500 dilution of HRP-conjugated polyclonal goat anti-human IgM (Chemicon, Melbourne Australia) in PBS with 0.1% Casein and 0.05% Tween 20 as the secondary antibody. HRP-conjugated monoclonal mouse anti-human IgG1 (IgG1 clone HP6069A, Calbiochem-Novabiochem Corp, CA USA) and HRP-conjugated monoclonal mouse anti-human IgG3 (IgG3 clone HP6047, Calbiochem-Novabiochem Corp, CA USA) were also used

at 1:2500 dilutions to detect IgG1 and IgG3 responses to AMA1(3D7). The specificity and sensitivity of the IgG1 and IgG3antibodies has been previously established (Stanisic et al. 2009).

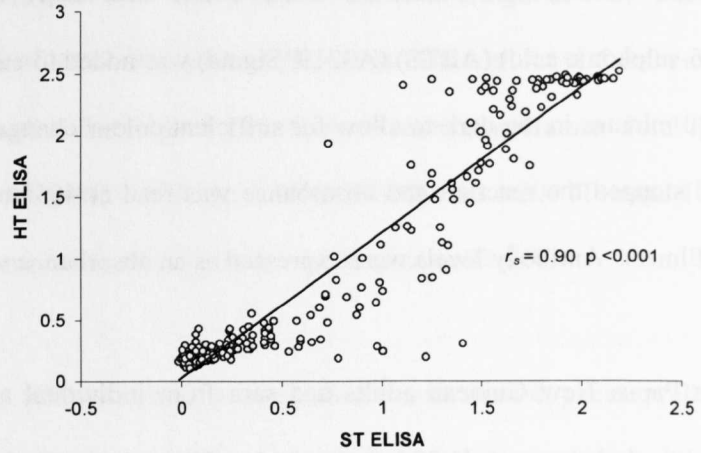

Figure 2.2 Correlation between standard ELISA (ST ELISA) and high throughput ELISA (HT ELISA). Spearman rank correlation, $r_s = 0.90$; $p < 0.001$; n = 272.

2.10.3 Competition ELISA

Antibodies to specific epitopes on AMA1 (3D7) were measured by a competitive ELISA method. Three monoclonal antibodies (1F9, 2C5, 5G8) with defined epitopes were used to compete with naturally acquired antibodies. Monoclonal antibody 1F9 inhibits invasion of 3D7 and D10 *P. falciparum* parasites, and recognises a polymorphic epitope in domain I (Coley et al. 2001; Coley et al. 2006). Monoclonal antibody 2C5 also reacts with 3D7 and D10 *P. falciparum* AMA1. Its epitope is conformational and requires the entire, correctly folded AMA1 ectodomain for reactivity (Coley et al. 2006). MAb 5G8 recognizes a non-strain specific19 residue linear epitope in the N-terminal pro-sequence/pro-domain of AMA1. The assay method was optimised with mAb 1F9 first then followed with the other two mAb. Optimisation for the concentration of monoclonal antibody (mAb) to use was carried out by using the serial dilution method.

ELISAs were carried out using Dynex Immunolon 4HBX 96 well plates (Dynex Technologies Inc) or Nunc Maxisorp 96 well plates (Nunc). Wells were coated with 100µl of 0.5µg/ml in PBS of antigen (3D7 AMA-1) and left overnight at 4°C. Wells were washed three times using a wash

buffer (PBS-Tween 20, 0.05%). 100µl/well of blocking buffer (PBS-Tween 20, 10% skimmed milk powder) was added to each well and the plates incubated at 37 °C for two hours. Serial concentrations mAbs 1F9, 2C5, and 5G8 were made up in sample buffer (PBS-Tween 20, 5% skimmed milk powder). After washing the plates once with wash buffer, 100µl/well of each concentration was added to duplicate wells and incubated at 37 °C for one hour. The plates were washed three times and incubated with 100µl/well of secondary antibody (HRP conjugated rabbit anti-mouse IgG) diluted at 1:2500 in sample buffer for one hour at room temperature. After three washes with wash buffer, 100µl/well of ABTS or OPD substrate solution was added. After 10 – 15 minutes incubation in the dark, the development reaction was stopped using 100µl/well 1% SDS (for use with ABTS) or 20µl/well 2M sulphuric acid (for use with the OPD substrate solution). The amount of antibody in each sample was then measured at 405nm (ABTS) or 492nm (OPD).

Standard curves for each mAb were then plotted and the optimum amount of mAb to compete with the serum samples was decided upon. A value close to saturation but still on the linear part of the curve was chosen so that there is enough mAb to occupy most specific sites in the absence of competing antibody. The amount of mAb chosen to add into subsequent assays was 0.2µg/ml of mAb 1F9 and mAb 2C5 and 0.12µg/ml of mAb 5G8. Serum samples were then tested for activity to the specific epitopes as stated above. Sample dilutions were 1:250 for 1F9 competition and 1:500 for 2C5 and 5G8 competition in sample buffer with mAb at the stated concentrations added. Reactivity was expressed as the percentage competition of the monoclonal antibody, with high values indicating high levels of epitope-specific antibodies in the serum sample.

2.11 Growth Inhibition Assay

Assays were performed by Fiona McCallum using established methods (Persson et al. 2006; McCallum et al. 2008). Heat inactivated dialysed sera was tested for inhibitory activity in 3077 sterile 96 well U-bottomed plates (Falcon) on highly synchronous *P. falciparum* 3D7 parasites. The majority of the parasites were at the late pigmented trophozoite stage. The cultures were

diluted with fresh erythrocytes to a starting parasitaemia 0.4% (for two cycle assays) and 0.7-0.8% (for one cycle assays). The erythrocytes were resuspended in culture medium to 1% hematocrit. 25µl of the parasite suspension was added to each well leaving the wells on the outside of the plate blank. Sterile PBS was added to the blank wells to keep the plate humid. 2.5µl of the serum sample was added in duplicate. Positive controls of 100µg/ml and 10 µg/ml pharmaceutical grade heparin and 1F9 monoclonal antibody (against 3D7 AMA-1) were added to each plate. PBS was added to act as a negative control. A reference smear from the remaining parasite sample used for the assay was later used to determine the parasitaemia and stage of culture. The plate was then put in a humidified chamber, gassed and incubated at 37°C. For a two cycle assay, the cultures were fed after 48 hours by adding 5µl of media into each well. Duplicate plates each containing the same serum samples but different transfected parasite clones were set up at the same time. After 36-48 hours (one cycle assay) or 78-84 hours (two cycle assay) when the parasites were at the late ring or early trophozoite stage, the parasites were stained in the following manner. 100µl of 10µg/ml ethidium bromide (molecular biology grade) in PBS was added to each well with gentle mixing and incubated in the dark for 1 hour. The plates were spun at 1500rpm for 1 min to pellet the erythrocytes. Supernatant was removed and the erythrocytes were resuspended in 200µl PBS and the parasitaemia measured by flow cytometry.

2.12 Statistical Analysis

Data was cleaned and all analyses performed with STATA version 9.2 (StataCorp, College Station, TX USA). Statistical significance was set at the conventional level ($p \leq 0.05$) for all tests. All OD readings were standardised using the positive controls on each plate. Standardisation was carried after the mean OD value of the blank wells was subtracted to remove the background. Using the same positive control samples that were present on each plate, a calculation of the conversion factor that would standardise the OD readings across all the plates was carried out as follows:

$$\text{Conversion Factor} = \frac{\text{Positive control OD}}{\text{Mean of all positive control ODs}}$$

Standardised OD values were then calculated by dividing the OD value of the test samples by the conversion factor. The cut-off value for antibody positivity to a particular antigen was calculated as any value above the mean+3SD above the value of all the negative controls on each plate. The cut-off value for a positive anti-AMA1 IgG response was the same for all three variants (0.10 OD units), and that for a positive anti-MSP2 response was 0.50 OD units for both variants tested. The cut-off values for anti-AMA1 (3D7) IgG1, IgG3, and IgM responses were 0.34 OD units, 0.07 OD units, and 0.14 OD units respectively.

2.12.1 Acquisition of antibodies to AMA1 and MSP2

The chi^2 test for trend was used to assess the acquisition of antibodies to AMA1 and MSP2 in different age groups and at different sample times, comparing the proportion of individuals who were antibody positive. As the antibody data was non-normally distributed, non-parametric methods (Kruskal-Wallis and Mann-Whitney tests) were used to compare antibody levels. Spearman rank correlation coefficients were used to examine the association between antibody levels to different antigens.

2.12.2 Antibody response ranking

Tracking is a method used to investigate the stability of outcome variables over time. Rather than calculating a tracking coefficient for each individual, a simpler method of visualizing relative change over time was used. Each individual's antibody response was assigned as a rank within the study population and its stability was investigated over the study period using adjusted *z*-scores (standard normal deviates). The *z*-score was calculated using the following equation:

$$z = \frac{\chi - \mu}{\sigma}$$

Where χ is the antibody measure, μ is the mean, and σ is the standard deviation. The *z*-score was adjusted for age and sample time to take into account the increasing age of the children over the study period and the reduction in malaria transmission.

2.12.3 Factors affecting antibody titres at sample time

To investigate the factors affecting antibody titres; univariate and multivariable linear regression was carried out with age (one year intervals), parasite status (binary), sickle trait (categorical), alpha-thalassemia (categorical), and prior parasitaemia/illness (categorical) as covariates. All the data was fitted into the multivariable model and then repeated after stratifying by parasite status at sampling time as this modifies the effects of antibodies to *P. falciparum* blood-stage antigens (Bull et al. 2002; Polley et al. 2004; Polley et al. 2006; Osier et al. 2008).

2.12.4 Locally Weighted Scatter-plot Smoothing

In order to reduce the large number of data points at each time point to a longitudinal pattern, mean antibody levels were calculated and graphed using locally weighted scatter-plot smoothing (LOWESS). LOWESS fits a low-degree polynomial to a determined subset of the data at each point with explanatory variable values near the point where the response is being estimated (Cleveland 1979; Cleveland and Devlin 1988). 80% of the data at each point was used to fit each local polynomial using weighted least squares which gives less weight to points further away from the point whose response is being estimated. LOWESS does not require the specification of a function to fit a model to all of the data in the sample and requires large densely sampled data sets to produce good models. With over 250 data points at each sample point, this model gave a reasonable population mean.

2.12.5 Probability of a high antibody response after recorded *P. falciparum* exposure

The probability of a high antibody response (above the 75th centile) to each antigen after recorded exposure to *P. falciparum* was estimated by logistic regression, fitting the number of recorded parasitemic episodes in the 6 months prior to sample time as a factor and adjusting for age. The logits from these models were converted into probabilities to give estimates of risk (Mitchell and Chen 2005). The probabilities were then plotted using the lowess smoothing technique to filter

fluctuations and highlight the overall trend. This same analysis was employed to estimate and plot the probability of being sero-positive to the various antigens.

2.12.6 Multivariate Analysis of Variance (MANOVA) for Repeated Measures

To test for differences in antibody levels over the study period MANOVA for repeated measures was carried out. This statistical test is carried out for the $T - 1$ absolute differences between subsequent measurements.

The F statistic for this test is calculated using the following equations:

$$F = \left(\frac{N - T + 1}{(N-1)(T-1)} \right) H^2$$

$$H^2 = \frac{N y'_d y_d}{S^2_d}$$

Where N is the number of subjects, T is the number of repeated measurements, y'_d is the row vector of differences between subsequent measurements, y_d is the column vector of differences between subsequent measurements, and S^2_d is the variance/covariance matrix of the differences between subsequent measurements. MANOVA for repeated measures assumes that observations on different subjects at each of the time points are independent and are multivariate normally distributed (multivariate normal distribution is an extension of normal one dimensional distribution – the assumption is that each linear combination of a random vector's components has a univariate normal distribution).

2.12.7 Generalised Estimating Equations (GEE)

To investigate the effect of different predictor variables (X) on antibody levels (continuous outcome variable, Y) generalised estimating equations (GEE) were used. GEE allow for the analysis of longitudinal relationships using all available longitudinal data without summarizing the longitudinal outcome into a single value (Twisk 2003). The model for GEE is an extension of cross-sectional linear regression and uses the formula below as described by Twisk:

$$Y_{it} = \beta_0 + \sum_{j=1}^{J} \beta_{1j} X_{itj} + \beta_2 t + \sum_{k=1}^{K} \beta_{3k} Z_{ikt} + \sum_{m=1}^{M} \beta_{4m} G_{im} + \varepsilon_{it}$$

Where Y_{it} are the observations for subject i at time t, β_0 is the intercept, X_{itj} is the independent variable j for subject i at time t, β_{1j} is the regression coefficient for independent variable j, J is the number of independent variables, t is time, β_2 is the regression coefficient for time, Z_{ikt} is the time-dependent covariate k for subject i at time t, β_{3k} is the regression coefficient for time-dependent covariate k, K is the number of time-dependent covariates, G_{im} is the time-independent covariate m for subject i, β_{4m} is the regression coefficient for time-independent covariate m, M is the number of time-independent covariates, and ε_{it} is the 'error' for subject i at time t. The estimated β_{1j} represents the relationship between the longitudinal development of the outcome variable (Y_{it}) and the development of different predictor variables (X_{it}). This model allows for simultaneous analysis between variables at different time points. Repeated observations on the same subject are not independent so a correction needs to be made for this within-subject correlation. An exchangeable correlation structure is the simplest correlation structure. Here, correlations between subsequent measurements are assumed to be the same irrespective of the length of the time interval. With correlation values ranging from 0.6 to 0.75, it was decided that the exchangeable correlation structure would work best with this dataset. This within-subject correlation structure is treated as a covariate.

The above equation then becomes:

$$Y_{it} = \beta_0 + \sum_{j=1}^{J} \beta_{1j} X_{itj} + \beta_2 t + \ldots + \mathrm{CORR}_{it} + \varepsilon_{it}$$

Where CORR_{it} is the working correlation structure.

The risk of experiencing a recorded episode of parasitaemia in the intervals was investigated using GEE for 'count' outcome variables. This model uses the same equations as above and is comparable to a poisson regression analysis except that within-subject correlation is taken into account. An exchangeable correlation structure was also used in this model. Antibody measures were entered into the model as continuous variables, age was a categorical variable defined by age at the start of the study, parasitaemia was also a categorical variable defined by the number of times

an individual had concurrent parasitaemia at sample time. Prior exposure was a count variable defined by the number of times each individual had an episode of recorded parasitaemia in six months prior to sample time. Sickle trait was entered as a binary variable (normal and heterozygous) and α-thalassemia as a categorical variable (normal, heterozygous, homozygous).

2.12.8 Antibody responses and protection against clinical malaria (Cross sectional risk analysis)

In order to investigate whether the risk of disease was dependent on the time that sampling occurred, univariate and multivariate generalised linear models (GLM) were used on samples taken during the May and October 2002 bleeds as well as those taken during the May 2003 bleeds. From October 2003 to October 2004 there were no recorded episodes of clinical malaria in this cohort so antibody measures for samples collected after the May 2003 bleed were not included in the analysis. Univariate analysis was first carried out to assess the non-antibody factors that affected the risk of clinical disease. These factors were age, concurrent parasitaemia, and haemoglobinopathies. Each was included in the model in different ways. Age was a continuous variable, concurrent parasitaemia was coded as both a binary variable (at sample time) and also as a categorical variable when it described an individual's ongoing parasitemic status over the study period (never parasitemic, parasitemic once, and recurrent parasitaemia). Sickle trait was a binary variable and alpha-thalassemia a categorical variable (normal, heterozygous, homozygous).

Univariate analysis was carried out on the May and October 2002 samples as well as the May 2003 samples as described above. It was then carried out on all the samples in the study using the same model but including a clustering indicator that identified repeated sampling on the same individuals but ignored the longitudinal nature of the sampling. Each antibody response was also tested as a continuous variable in the univariate models described above. Multivariable GLM analysis for the risk of a clinical episode was carried out on the antibody responses for the May 2002 to May 2003 bleeds as well as for all samples and was adjusted for age, concurrent parasitaemia, and haemoglobinopathies.

2.12.9 Antibody responses and protection against clinical malaria (Longitudinal risk analysis)

The overall risk of a clinical episode of malaria in this study was assessed using Generalised Estimating Equations (GEE) for 'count' outcome variables. Univariate GEE analysis on antibody responses and the non-antibody factors that may affect the risk of clinical malaria was carried out followed by multivariable analysis on the adjusted effect of antibody responses to AMA1 and MSP2. Recently published results from a separate study carried out in Kilifi has shown that removal of those individuals without any exposure strengthened the protective association of some malarial antigens against clinical disease (Polley et al. 2004; Bejon et al. 2009). With this in mind, I carried out the multivariable analysis on only those children who had recorded exposure to *Plasmodium* over the study period (as indicated by a positive blood smear during the weekly surveillance or at sample time).

2.12.10 Acquisition of antibody responses to specific epitopes of AMA1(3D7)

Differences in median antibody levels were analysed using the Kruskal-Wallis test. Tests for trend were used to check for patterns in the proportion of the population acquiring a particular response. Antibody levels to recombinant AMA1 (3D7), the 1F9, 2C5, 5G8 epitopes, and parasite growth *in vitro* were compared using Spearmans rank correlation.

2.12.11 Association between epitope-specific antibody responses and rate of clinical malaria

In the Chonyi cohort (chapter 7), Kaplan-Meier survival functions were used to investigate the failure rates and characteristics of the groups described above. Differences in survival functions were tested using the Log-rank test. Differences in the likelihood of failure at any point during follow up between high and low responders was analysed using Cox Proportional Hazards. Both the unadjusted and age adjusted proportional hazards were investigated.

2.12.12 Epitope-specific antibody responses and protection against clinical malaria

The relationship between antibody levels and the risk of experiencing a clinical malaria episode in the 6 months post-sampling for the Chonyi cohort (chapter 7) was analysed using univariate and then multivariable generalised linear models (GLM) that were adjusted for age (in 1 year categories). Risk was expressed as a risk ratio as the outcome incidence was above 10%; and was compared between those with high and low antibody responses (assigned using the median value) in the entire cohort as well as in groups stratified by parasite status.

2.13 Cohort descriptions

Following are details of the characteristics of each of the cohorts used in this study.

2.13.1 Longitudinal Ngerenya Cohort

As described earlier, this study covers the period from May 2002 to October 2004. In total 1804 samples were analysed, 750 (44.6%) from females and 933 (55.4%) from males. There was an average of 300 children per sampling point with 186 of them present at all six sampling points. The gender distribution remained constant throughout the study (Table 2.1). This was an aging cohort with the median age being 3.7 years in May2003 and 5.3 years in October 2004 (Table 2.2). At each sampling point the age ranged from 0 to 9 years. Of the 1728 total samples where blood smear data was available, 1550 (89.7%) were aparasitemic and 178 (10.3%) parasitemic at sampling. Malaria transmission was on the decline over the latter part of the study as evidenced by the number of children with parasites at each cross-sectional bleed. Parasite prevalence fell from 17.1% in May 2002 to 3.7% in October 2004 (Table 2.1).

Table 2.1 Gender and parasite distribution at each cross sectional bleed (longitudinal study)

Bleed	Gender % (n)			Parasite status % (n)		
	n	Female	Male	n	Negative	Positive
May 2002	309	44.5 (133)	55.5 (166)	299	82.9 (248)	17.1 (51)
October 2002	295	44.4 (128)	55.6 (160)	292	92.8 (271)	7.2 (21)
May 2003	296	44.5 (130)	55.5 (162)	288	86.8 (250)	13.2 (38)
October 2003	300	44.5 (125)	55.5 (156)	294	86.0 (253)	14.0 (41)
May2004	302	44.4 (115)	55.6 (144)	282	94.0 (265)	6.0 (17)
October 2004	302	45.1 (119)	54.9 (145)	273	96.3 (263)	3.7 (10)
All	**1804**	**44.6 (750)**	**55.4 (933)**	**1728**	**89.7 (1550)**	**10.3 (178)**

There were minor increases in prevalence that were sustained in 2003 with prevalence rates of 13.2% in May and 14.0% in October. The decline in malaria transmission was also seen in the frequency of positive blood smears and clinical malaria episodes in the intervals between cross-sectional sampling (Table 2.3). During the six-month interval before the first sampling in May 2002, 34.4% children experienced at least one parasitemic episode. Of these children, 52.4% had at least one clinical mild malaria episode. Between the May 2002 and October 2002 sampling 17.3% had one or more parasitemic episodes. Malaria transmission increased in the following six months (October 2002 to May 2003) with 52% of the children experiencing parasitaemia. It then fell steadily in the three six-month intervals between May 2003 and October 2004 from 37% to 12.5% and finally to 2.6%. From October 2003 there were no clinical malaria episodes (Table 2.3). Sickle trait and α-thalassemia prevalence data was also available for this cohort. 13.1% of the children had the sickle trait (HbAS) compared to 86.9% of the normal (HbAA) phenotype. 18.4% of the children were homozygous for α-thalassemia compared to 48.6% heterozygotes and 33.0% normal children.

Table 2.2 Mean and median age at each cross sectional bleed (longitudinal study)

Bleed	n	Age (years)	
		Mean (SD)	Median (IQR)
May 2002	299	3.8 (2.1)	3.7 (2.0, 5.6)
October 2002	292	4.0 (2.3)	3.9 (2.1, 5.9)
May 2003	288	4.4 (2.4)	4.3 (2.4, 6.4)
October 2003	295	4.6 (2.5)	4.6 (2.6, 6.8)
May 2004	281	4.9 (2.6)	4.9 (2.8, 7.1)
October 2004	273	5.2 (2.7)	5.3 (2.8, 7.6)

Table 2.3 Proportion of children experiencing different numbers of episodes of parasitaemia and mild malaria in the intervals between cross-sectional sampling points (longitudinal study)

Number of episodes[1]	Proportion % (n)	
	Parasitaemia[†]	Mild malaria[Δ]
October 2001 – May 2002 (n=305)		
0	65.6 (200)	82.0 (250)
1	21.0 (67)	15.4 (47)
2	8.9 (27)	1.6 (5)
3	2.6 (8)	1.0 (3)
4	0.7 (2)	-
5	0.3 (1)	-
May 2002 – October 2002 (n=255)		
0	82.8 (211)	91.4 (233)
1	12.9 (33)	6.7 (17)
2	3.9 (10)	1.6 (4)
3	-	-
4	0.4 (1)	0.4 (1)
October 2002 – May 2003 (n=256)		
0	48.1 (123)	61.3 (157)
1	23.4 (60)	23.1 (59)
2	16.8 (43)	10.2 (26)
3	5.5 (14)	4.7 (12)
4	5.1 (13)	0.4 (1)
5	0.4 (1)	0.4 (1)
6	-	-
7	0.8 (2)	-
May 2003 – October 2003 (n=262)		
0	63.0 (165)	76.7 (201)
1	21.4 (56)	17.9 (47)
2	10.7 (28)	4.2 (11)
3	4.2 (11)	1.2 (3)
4	0.8 (2)	-
October 2003 – May 2004 (n=256)		
0	87.5 (224)	100 (256)
1	9.8 (25)	-
2	2.3 (6)	-
3	0.4 (1)	-
May 2004 – October 2004 (n=235)		
0	97.5 (229)	100 (235)
1	2.1 (5)	-
2	0.4 (1)	-

Notes

[1] Number of episodes recorded during weekly surveillance in the interval between cross sectional bleeds.

[†] Recorded parasitaemia accompanied by fever ≥ 37.5°C.

[Δ] Malaria definitions: detectable parasitaemia and fever ≥ 37.5°C in children < 1 year, parasitaemia ≥ 2500/µl and fever ≥ 37.5°C in children ≥ 1 year.

2.13.2 Ngerenya September 1998 Cohort

130 serum samples collected in September 1998 from Ngrerenya residents aged 0-85 years were used in this study. 53.4% were female and 45.7% were male. Data was analysed in five age groups (0-4, 5-8, 9-12, and 13+). 39.3% of the cohort was parasitemic at sampling (Table 2.4)

Table 2.4 Parasite status and age distribution in the Ngerenya September 1998 cohort

Age group (years)	n	Parasite Status % (n)	
		Positive	Negative
0 -	29	27.6 (8)	72.4 (21)
5 -	40	57.5 (23)	42.5 (17)
9 -	30	53.3 (16)	46.7 (14)
13 +	51	23.5 (12)	76.5 (39)
All	**150**	**39.3 (59)**	**60.7 (91)**

2.13.3 Chonyi October 2000 Cohort

In total 276 serum samples from Chonyi were collected in October 2000 from individuals 0-55 years of age. 51.4% were female and 48.6% were male. All individuals were monitored weekly by active and passive surveillance for symptomatic illness and malaria. 42.6% of the samples were from individuals with concurrent parasitaemia (Table 2.6). More than half of the samples from individuals 7 years of age or older were parasitemic at sampling.

Table 2.5 Parasite status and age distribution in the Chonyi October 2000 cohort

Age group (years)	n	Parasite Status % (n)	
		Positive %	Negative %
0 -	20	10.0 (2)	90.0 (18)
1 -	51	29.4 (15)	70.6 (36)
3 -	52	46.2 (24)	53.9 (28)
5 -	52	46.2 (24)	53.9 (28)
7 -	53	50.9 (27)	49.1 (26)
9 -	20	90.0 (18)	10.0 (2)
11 +	27	25.9 (7)	74.1 (20)
All	**275**	**42.6 (117)**	**57.5 (158)**

3 Identification of factors determining the acquisition and levels of antibodies to AMA1 and MSP2, and the influence of changing malaria transmission intensity

3.1 Introduction

The presence of malaria antigen specific antibodies in endemic populations is an important correlate of immunity (Riley et al. 1992; Hogh et al. 1995; Al-Yaman et al. 1996; Egan et al. 1996; Branch et al. 1998; Conway et al. 2000; Polley et al. 2003; Cavanagh et al. 2004; Perraut et al. 2005; Dodoo et al. 2008; Osier et al. 2008; Stanisic et al. 2009; Fowkes et al. 2010). That antibody levels rise with age – often reaching saturation in adulthood – along with the rarity of malaria-related deaths in adults in populations exposed to endemic malaria indicates that protective antibody responses are acquired with cumulative exposure. In most areas of sub-Saharan Africa, the most active phase of immunity acquisition occurs in children below the age of 10 years, after which malaria related morbidity becomes rare. It is hypothesised that immunity to severe malaria is acquired after a small number of episodes, followed by broader immunity to mild malaria, and finally anti-parasite immunity (Marsh and Snow 1997; Gupta et al. 1999).

The overwhelming evidence of increasing antibody responses with age – both in magnitude and prevalence – that has been produced by a large body of serological studies often leads to over simplification of the understanding of the antibody acquisition process in childhood. One of the tenets in malaria immunology is that antibodies will always increase with age in malaria endemic populations as individuals get more exposure to the pathogen. This is typically seen in cross-sectional studies and sometimes leads to age being used as a proxy of exposure which is corrected for in protective association studies. Although it is acknowledged that age and exposure are two related factors associated with immunity, only a few studies have successfully separated the two and shown that age has an independent effect on antibody responses (Baird et al. 1991; Kurtis et al. 2001; Hudson Keenihan et al. 2003; Keenihan et al. 2003). These studies compared the antibody

responses in adults and children; to date there is no study that investigates age independently of exposure at the time when acquisition of immunity to malaria is at its highest rate (childhood) in malaria endemic populations.

Antibody titres increase with increasing endemicity and drop significantly after an episode of severe malaria (Jakobsen et al. 1997; Kinyanjui et al. 2007). In areas of seasonal malaria transmission, antibody levels appear to be maintained as long as parasite exposure continues (Fruh et al. 1991; Ramasamy et al. 1994; Cavanagh et al. 1998; Soares et al. 1999b; John et al. 2002). These two observations are evidence of a differential antibody induction rate which is poorly described in the published malaria literature. Malaria in Africa is currently typified by both a reduction in malaria transmission in parts as well as a wide range of malaria transmission intensities (Hay et al. 2000; Bhattarai et al. 2007; Fegan et al. 2007; O'Meara et al. 2008; Okiro et al. 2009). Classic models of antibody acquisition in childhood assume fixed transmission intensities which are unlikely to capture this dynamic effect, and often assume highly stable antibody levels.

Antibody levels are higher in parasitized individuals and can drop more than 10-fold in the absence of parasites (Soares et al. 1997; Soares et al. 1999a). But even though antibody prevalence is associated with parasitaemia, the significant increase is seen predominantly in children rather than adults (Al-Yaman et al. 1994; Al-Yaman et al. 1995b). Studies of the association of antibody responses with protection from clinical disease often stratify by parasite infection status in order to separate the effect of circulating parasites on antibody responses (Bull et al. 2002; Polley et al. 2004). Though this may help with defining an individual's overall responsiveness, it does not give us much detail into whether the response we are measuring is made up of the response to both the cumulative and current exposure or whether it is an overwhelming response to the concurrent parasitaemia.

Two haemoglobinopathies – sickle trait, and alpha-thalassemia – affect the incidence of malaria in endemic populations. The sickle trait (HbAS) confers a considerable resistance to severe and

complicated malaria in children below the age of five years as well as to mild symptomatic malaria (Willcox et al. 1983; Hill et al. 1991; Aidoo et al. 2002; Williams et al. 2005b). Up-regulation of cell-mediated responses to malaria antigens in HbAS individuals from Sudan and the Gambia has been observed (Marsh et al. 1989; Bayoumi et al. 1990; Abu-Zeid et al. 1992). That protection from clinical malaria increases with age at a more rapid pace in HbAS children than in HbAA children also implies a role for HbAS in regulation of the adaptive immune response (Williams et al. 2005a). Though two studies have shown differences in antibody responses between HbAA and HbAS individuals, they have not been in complete agreement (Sarr et al. 2006; Verra et al. 2007). Due to the paucity of results, it remains unclear whether the presence of the sickle trait leads to a reduction or increase in the antibody response to malaria antigens.

Studies in Papua New Guinea and Kenya showed that homozygotes for alpha-thalassemia were more protected than normal individuals from severe malaria (Allen et al. 1997; Williams et al. 2005c). This was contrary to a smaller study in Ghana which showed the same protective effect in heterozygotes but not in homozygotes (Mockenhaupt et al. 2004). Although inhibition of parasite growth and invasion have been suggested as mechanisms for the protective action of alpha-thalassemia, this seems unlikely as the parasite densities measured in the Kenyan and Papua New Guinean studies were no different in homozygotes and normal individuals, and there is no consistent protective association observed for mild symptomatic malaria (Williams et al. 2005c; Wambua et al. 2006; Fowkes et al. 2008). Though the mode of action of protection attributed to antibodies with regards to alpha-thalassemia remains unclear, there still remains a dearth of basic information on the acquisition of antibody responses in individuals with alpha-thalassemia.

To date, there is an insufficient amount of information on the direct effect that the above mentioned factors have on antibody levels. This type of information is important for thorough interpretation of antibody responses that are often measured in malaria-endemic populations and interpreted without detailed knowledge of which factors have been instrumental in giving the result obtained. When antibody responses begin to be seen as a result of other factors that may be dynamic in nature rather than simply a baseline measurement of immunity, one can see why there is conflicting

data in studies associating antibody responses with protection from malaria. The association of antibody responses with protection is beyond the scope of this chapter and will be discussed in later chapters. In this chapter I will identify factors and quantify their effect on the acquisition and maintenance of antibody responses to merozoite antigens in children from Kilifi district in Kenya. Using a unique cohort with repeated sampling that covered a period when malaria transmission was on the decline (parasite prevalence dropped from 17% in May 2002 to 4% in October 2004 and incidence of mild malaria from 12% in between October 2001 and May 2002 to 0% from October 2003 onwards), I will compare the acquisition of antibody responses to various alleles of AMA1 and MSP2 as representative merozoite antigens.

3.2 Methods

3.2.1 Study population, sample collection, and surveillance

Serum samples were collected from an observational longitudinal cohort study with 6 sampling points approximately six months apart from May 2002 to October 2004 with continuous active weekly case detection, as described in chapter 2.

3.2.2 Enzyme-linked immunosorbent assay (ELISA)

Total IgG responses to recombinant proteins representing three AMA1 variants (W2mef, HB3, and 3D7) and two MSP2 variants (3D7 and FC27) were measured by high throughput ELISA according to the protocol described in chapter 2. IgG responses to schizont extract and tetanus toxoid were also measured by ELISA. Antibodies were tested at sera concentrations of 1:4000 (AMA1 variants), 1:1000 (MSP2 variants and schizont protein extract), and 1:500 (tetanus toxoid).

3.2.3 Statistical Analysis

All data analyses were performed with STATA version 9.2 (Stata Corp, College Station, TX USA). All samples were used in the analyses unless stated. The chi^2 test for trend was used to assess the acquisition of antibodies to AMA1 and MSP2 in different age groups and at different sample times. As the antibody data was non-normally distributed, non-parametric methods (Kruskal-Wallis and Mann-Whitney tests) were used to compare antibody titres. Spearman rank correlation coefficients were used to examine the association between antibody titres to different antigens. Significance was reached at the 0.05 level for all tests.

To investigate the factors affecting antibody titres; univariate and multivariable linear regression was carried out with age, parasite infection status, sickle trait, alpha-thalassemia, and prior parasitaemia or prior illness as covariates. All the data was fitted into the multivariable model and then repeated after stratifying by parasite status as concurrent parasitaemia at sample time modifies the effects of antibodies to *P. falciparum* blood-stage antigens (Bull et al. 2002; Polley et al. 2004; Polley et al. 2006; Osier et al. 2008).

The probability of a high antibody response (above the 75^{th} centile) to each antigen after recorded exposure to *P. falciparum* was estimated by logistic regression, fitting the number of recorded parasitaemic episodes in the 6 months prior to sample time as a factor. These odds were converted into the probability of a high antibody response (Mitchell and Chen 2005). The probabilities were then plotted using the LOWESS smoothing technique to filter fluctuations and highlight the overall trend. This same analysis was employed to estimate and plot the probability of being sero-positive to the various antigens.

3.3 Results

3.3.1 The prevalence and magnitude of IgG responses to AMA1 and MSP2 increase with age

Overall sero-prevalence of the IgG response to AMA1 and MSP2 variants was investigated using cut-off values determined by a panel of negative controls. Children were grouped according to their age at sampling and the sero-prevalence was determined at each bleed separately. More than 15% of all children below the age of one had detectable IgG responses to the AMA1 variants tested (W2mef, HB3, 3D7) (Tables 3.1-3.3). This was in contrast to sero-prevalence values ranging from 0 to 13% in May 2002 (MSP2(FC27)) with regards to the IgG response to MSP2 variants (3D7 and FC27) in children less than a year old (Tables 3.4-3.5). The prevalence of detectable antibodies then dropped in one year-olds before increasing with age in the same manner for all three AMA1 variants, with over 70% sero-positivity by 9 years of age (Tables 3.1-3.3). The prevalence of antibodies to both MSP2 variants in one year-olds remained similar to that in children below one year and did not begin to rise until 3 years of age (Tables 3.4-3.5). From that age there was a steady increase in antibody prevalence but only 37.5% (95% CI: 12.9 -62.2%) and 50% (95% CI: 24.6 -75.5%) of 9 year olds had detectable antibodies to MSP2(3D7) and MSP2(FC27) in May 2004 respectively, and only 28% (95% CI: 10-46.1%) and 36%(95% CI: 16.8-55.3%) in October 2004 respectively (Tables 3.4-3.5). Overall, there was a significant increase ($p \leq 0.05$, chi^2 test for trend) in the prevalence of detectable antibodies to AMA1 and MSP2 variants with increasing age. Antibodies to AMA1 appeared to be acquired at a faster rate and were more prevalent than those to MSP2 (Tables 3.1-3.5). Maternal antibodies to AMA1 variants appeared to be more prevalent than those to MSP2 (as indicated by the prevalence in children below the age of one year). Representative graphs of the prevalence of IgG responses to each antigen in May 2002 also showed the slower acquisition of antibody responses to MSP2 variants in comparison to that of AMA1 variants (Figure 3.1).

Table 3.1 Prevalence of detectable IgG responses to AMA1 (W2mef) by age and cross sectional bleed in the Ngerenya cohort.

Age (years)	Anti-AMA1(W2mef) IgG sero-prevalence (95% CI)						
	May 2002[1]	October 2002[1]	May 2003[1]	October 2003[1]	May 2004[1]	October 2004[1]	*P*-value[2]
0	26.7 (10.6 - 42.9)	67.6 (52.3 - 83)	26 (9.1 - 42.9)	43.5 (22.7 - 64.3)	19.1 (1.8 - 36.4)	33.4 (8.6 - 58.2)	0.182
1	14.6 (4.5 - 24.8)	21.9 (7.3 - 36.5)	34.7 (15.9 - 53.4)	11.8 (0.8 - 22.9)	16 (1.3 - 30.8)	8.4 (-3.1 - 19.7)	0.385
2	33.4 (18.3 - 48.4)	48.8 (32.8 - 64.7)	11.4 (1.9 - 20.9)	15.7 (2.8 - 28.5)	6.5 (-2.4 - 15.3)	3.2 (-3.1 - 9.3)	**<0.001**
3	48.8 (33.3 - 64.4)	59 (43.3 - 74.7)	34.4 (17.6 - 51.2)	43.3 (27 - 59.5)	32.5 (17.1 - 47.8)	17.9 (3.4 - 32.4)	**0.002**
4	50 (32.4 - 67.7)	76.7 (61.3 - 92.2)	40 (24.6 - 55.5)	47.7 (32.3 - 63)	17.3 (3.2 - 31.3)	33.4 (16.1 - 50.6)	**0.001**
5	61.8 (47.6 - 75.9)	71.5 (58.6 - 84.3)	48.3 (29.7 - 66.9)	46.2 (26.6 - 65.8)	45.5 (28.2 - 62.8)	42.5 (25.3 - 59.7)	**0.007**
6	65.8 (50.5 - 81.2)	75.9 (60 - 91.8)	52.8 (36.2 - 69.4)	62 (47 - 76.9)	54.2 (33.8 - 74.7)	41.7 (21.5 - 62)	**0.032**
7	74 (55.5 - 92.4)	82.9 (70.2 - 95.6)	71 (54.7 - 87.3)	52 (32 - 72.1)	57.9 (42 - 73.9)	55.3 (39.2 - 71.4)	**0.007**
8[¢]	-	100 (0 - 0)	75 (55.5 - 94.6)	81.9 (68.4 - 95.3)	72 (54 - 90.1)	41.7 (21.5 - 62)	**0.005**
9[¢]	-	-	-	-	81.3 (61.5 - 101.1)	72 (54 - 90.1)	0.506
P-value[3]	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	

Notes

[1] Cross sectional bleed.

[2] *P*-values calculated using a chi^2 test for trend for IgG prevalence in the same age group at different cross sectional bleeds (*P*-values ≤ 0.05 indicated in bold type).

[3] *P*-values calculated using a chi^2 test for trend for IgG prevalence in different age groups at the same cross sectional bleed (*P*-values ≤ 0.05 indicated in bold type).

[¢] Missing values are due to a lack of children in that age group.

All samples included in analysis.

Table 3.2 Prevalence of detectable IgG responses to AMA1 (HB3) by age and cross sectional bleed in the Ngerenya cohort.

Age (years)	Anti-AMA1(HB3) IgG sero-prevalence (95% CI)						
	May 2002[1]	October 2002[1]	May 2003[1]	October 2003[1]	May 2004[1]	October 2004[1]	*P*-value[2]
0	26.7 (10.6 - 42.9)	56.8 (40.6 - 73.1)	37.1 (18.4 - 55.7)	34.8 (14.8 - 54.8)	23.9 (5.1 - 42.6)	33.4 (8.6 - 58.2)	0.361
1	14.6 (4.5 - 24.8)	9.4 (-1 - 19.7)	34.7 (15.9 - 53.4)	14.8 (2.6 - 26.9)	12 (-1.1 - 25.1)	8.4 (-3.1 - 19.7)	0.619
2	33.4 (18.3 - 48.4)	20.6 (7.7 - 33.5)	25 (12.1 - 38)	21.9 (7.3 - 36.5)	9.7 (-1 - 20.4)	3.2 (-3.1 - 9.3)	**0.001**
3	46.4 (30.9 - 61.9)	46.2 (30.3 - 62.1)	31.3 (14.9 - 47.7)	43.3 (27 - 59.5)	32.5 (17.1 - 47.8)	17.9 (3.4 - 32.4)	**0.017**
4	53.2 (35.5 - 70.8)	66.7 (49.5 - 83.9)	55 (39.4 - 70.7)	45.3 (30 - 60.6)	27.6 (11 - 44.3)	30 (13.3 - 46.8)	**0.002**
5	68.1 (54.6 - 81.7)	57.2 (43.1 - 71.3)	62.1 (44.1 - 80.2)	46.2 (26.6 - 65.8)	45.5 (28.2 - 62.8)	39.4 (22.4 - 56.4)	**0.004**
6	79 (65.8 - 92.2)	55.2 (36.7 - 73.7)	61.2 (44.9 - 77.4)	57.2 (42 - 72.4)	54.2 (33.8 - 74.7)	37.5 (17.7 - 57.4)	**0.004**
7	74 (55.5 - 92.4)	77.2 (63 - 91.4)	83.9 (70.7 - 97.1)	52 (32 - 72.1)	52.7 (36.5 - 68.8)	52.7 (36.5 - 68.8)	**0.002**
8[¢]	-	100 (0 - 0)	85 (68.9 - 101.2)	84.9 (72.4 - 97.4)	80 (64 - 96.1)	45.9 (25.4 - 66.3)	**0.002**
9[¢]	-	-	-	-	75 (53 - 97.1)	76 (58.9 - 93.2)	0.943
P-value[3]	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	

Notes

[1] Cross sectional bleed.

[2] *P*-values calculated using a chi^2 test for trend for IgG prevalence in the same age group at different cross sectional bleeds (*P*-values ≤ 0.05 indicated in bold type).

[3] *P*-values calculated using a chi^2 test for trend for IgG prevalence in different age groups at the same cross sectional bleed (*P*-values ≤ 0.05 indicated in bold type).

[¢] Missing values are due to a lack of children in that age group.

All samples included in analysis.

Table 3.3 Prevalence of detectable IgG responses to AMA1 (3D7) by age and cross sectional bleed in the Ngerenya cohort.

Age (years)	Anti-AMA1(3D7) IgG sero-prevalence (95% CI)						
	May 2002[1]	October 2002[1]	May 2003[1]	October 2003[1]	May 2004[1]	October 2004[1]	*P*-value[2]
0	23.4 (7.9 - 38.8)	48.7 (32.3 - 65.1)	29.7 (12.1 - 47.3)	26.1 (7.7 - 44.6)	23.9 (5.1 - 42.6)	26.7 (3.4 - 50)	0.384
1	8.4 (0.4 - 16.3)	3.2 (-3.1 - 9.3)	34.7 (15.9 - 53.4)	20.6 (6.8 - 34.5)	8 (-3 - 19)	12.5 (-1.1 - 26.1)	0.386
2	35.9 (20.6 - 51.3)	20.6 (7.7 - 33.5)	13.7 (3.4 - 24)	18.8 (5 - 32.6)	13 (0.9 - 25)	6.3 (-2.4 - 14.9)	**0.002**
3	46.4 (30.9 - 61.9)	35.9 (20.6 - 51.3)	28.2 (12.3 - 44.1)	37.9 (22 - 53.8)	29.8 (14.8 - 44.8)	17.9 (3.4 - 32.4)	**0.027**
4	40.7 (23.3 - 58)	63.4 (45.8 - 81)	47.5 (31.8 - 63.3)	40.5 (25.4 - 55.6)	17.3 (3.2 - 31.3)	33.4 (16.1 - 50.6)	**0.017**
5	63.9 (49.9 - 77.8)	49 (34.8 - 63.2)	51.8 (33.2 - 70.4)	46.2 (26.6 - 65.8)	39.4 (22.4 - 56.4)	30.4 (14.4 - 46.3)	**0.003**
6	71.1 (56.4 - 85.8)	55.2 (36.7 - 73.7)	50 (33.4 - 66.7)	57.2 (42 - 72.4)	41.7 (21.5 - 62)	33.4 (14 - 52.7)	**0.004**
7	65.3 (45.3 - 85.3)	68.6 (53 - 84.3)	77.5 (62.4 - 92.5)	52 (32 - 72.1)	50 (33.9 - 66.2)	50 (33.9 - 66.2)	**0.022**
8[6]	-	100 (0 - 0)	75 (55.5 - 94.6)	87.9 (76.6 - 99.3)	76 (58.9 - 93.2)	41.7 (21.5 - 62)	**0.003**
9[6]	-	-	-	-	81.3 (61.5 - 101.1)	72 (54 - 90.1)	0.506
P-value[3]	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	

Notes

[1] Cross sectional bleed.

[2] *P*-values calculated using a chi^2 test for trend for IgG prevalence in the same age group at different cross sectional bleeds (*P*-values ≤ 0.05 indicated in bold type).

[3] *P*-values calculated using a chi^2 test for trend for IgG prevalence in different age groups at the same cross sectional bleed (*P*-values ≤ 0.05 indicated in bold type).

[6] Missing values are due to a lack of children in that age group.

All samples included in analysis.

Table 3.4 Prevalence of detectable IgG responses to MSP2(3D7) by age and cross sectional bleed in the Ngerenya cohort.

Age (years)	Anti-MSP2(3D7) IgG sero-prevalence (95% CI)						
	May 2002[1]	October 2002[1]	May 2003[1]	October 2003[1]	May 2004[1]	October 2004[1]	*P*-value[2]
0	10 (-1 - 21)	2.8 (-2.7 - 8.1)	3.8 (-3.6 - 11)	0 (0 - 0)	0 (0 - 0)	6.7 (-6.5 - 19.8)	0.246
1	10.5 (1.7 - 19.2)	3.2 (-3.1 - 9.3)	3.9 (-3.8 - 11.5)	0 (0 - 0)	4 (-3.9 - 11.9)	4.2 (-4.1 - 12.4)	0.150
2	15.4 (3.9 - 27)	0 (0 - 0)	6.9 (-0.8 - 14.4)	3.2 (-3.1 - 9.3)	6.5 (-2.4 - 15.3)	3.2 (-3.1 - 9.3)	0.156
3	31.8 (17.3 - 46.2)	10.3 (0.6 - 20)	0 (0 - 0)	16.3 (4.2 - 28.4)	13.6 (2.3 - 24.8)	3.6 (-3.5 - 10.7)	**0.013**
4	18.8 (5 - 32.6)	13.4 (1 - 25.8)	20 (7.4 - 32.7)	19.1 (7 - 31.2)	6.9 (-2.6 - 16.4)	10 (-1 - 21)	0.249
5	49 (34.5 - 63.5)	14.3 (4.4 - 24.3)	13.8 (1 - 26.7)	3.9 (-3.8 - 11.5)	9.1 (-1 - 19.1)	6.1 (-2.3 - 14.4)	**<0.001**
6	47.4 (31.3 - 63.6)	27.6 (11 - 44.3)	27.8 (12.9 - 42.7)	31 (16.8 - 45.2)	8.4 (-3.1 - 19.7)	0 (0 - 0)	**<0.001**
7	52.2 (31.3 - 73.2)	20 (6.5 - 33.6)	22.6 (7.6 - 37.7)	28 (10 - 46.1)	23.7 (10 - 37.5)	10.6 (0.6 - 20.5)	**0.009**
8[6]	-	0 (0 - 0)	30 (9.4 - 50.7)	27.3 (11.8 - 42.8)	28 (10 - 46.1)	25 (7.3 - 42.8)	0.860
9[6]	-	-	-	-	37.5 (12.9 - 62.2)	28 (10 - 46.1)	0.529
P-value[3]	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	

Notes

[1] Cross sectional bleed.

[2] *P*-values calculated using a chi^2 test for trend for IgG prevalence in the same age group at different cross sectional bleeds (*P*-values ≤ 0.05 indicated in bold type).

[3] *P*-values calculated using a chi^2 test for trend for IgG prevalence in different age groups at the same cross sectional bleed (*P*-values ≤ 0.05 indicated in bold type).

[6] Missing values are due to a lack of children in that age group.

All samples included in analysis.

Table 3.5 Prevalence of detectable IgG responses to MSP2(FC27) by age and cross sectional bleed in the Ngerenya cohort.

Age (years)	Anti-MSP2(FC27) IgG sero-prevalence (95% CI)						
	May 2002[1]	October 2002[1]	May 2003[1]	October 2003[1]	May 2004[1]	October 2004[1]	*P*-value[2]
0	13.4 (1 - 25.8)	5.5 (-2.1 - 12.9)	7.5 (-2.8 - 17.6)	4.4 (-4.3 - 13)	4.8 (-4.7 - 14.2)	0 (0 - 0)	0.113
1	18.8 (7.6 - 30)	6.3 (-2.4 - 14.9)	7.7 (-2.8 - 18.2)	0 (0 - 0)	0 (0 - 0)	8.4 (-3.1 - 19.7)	**0.012**
2	12.9 (2.2 - 23.5)	10.3 (0.6 - 20)	6.9 (-0.8 - 14.4)	3.2 (-3.1 - 9.3)	6.5 (-2.4 - 15.3)	0 (0 - 0)	**0.026**
3	31.8 (17.3 - 46.2)	15.4 (3.9 - 27)	9.4 (-1 - 19.7)	24.4 (10.3 - 38.4)	16.3 (4.2 - 28.4)	3.6 (-3.5 - 10.7)	**0.026**
4	18.8 (5 - 32.6)	40 (22.1 - 58)	20 (7.4 - 32.7)	21.5 (8.9 - 34.1)	6.9 (-2.6 - 16.4)	10 (-1 - 21)	**0.024**
5	23.5 (11.2 - 35.7)	18.4 (7.4 - 29.4)	20.7 (5.7 - 35.8)	23.1 (6.5 - 39.7)	9.1 (-1 - 19.1)	6.1 (-2.3 - 14.4)	**0.031**
6	31.6 (16.6 - 46.7)	20.7 (5.7 - 35.8)	27.8 (12.9 - 42.7)	26.2 (12.7 - 39.8)	0 (0 - 0)	4.2 (-4.1 - 12.4)	**0.003**
7	39.2 (18.7 - 59.7)	34.3 (18.3 - 50.4)	35.5 (18.3 - 52.7)	20 (4 - 36.1)	10.6 (0.6 - 20.5)	10.6 (0.6 - 20.5)	**0.000**
8[¢]	-	0 (0 - 0)	50 (27.5 - 72.6)	45.5 (28.2 - 62.8)	36 (16.8 - 55.3)	12.5 (-1.1 - 26.1)	**0.012**
9[¢]	-	-	-	-	50 (24.6 - 75.5)	36 (16.8 - 55.3)	0.381
P-value[3]	**0.033**	**0.004**	**<0.001**	**<0.001**	**<0.001**	**0.001**	

Notes

[1] Cross sectional bleed.

[2] *P*-values calculated using a chi^2 test for trend for IgG prevalence in the same age group at different cross sectional bleeds (*P*-values ≤ 0.05 indicated in bold type).

[3] *P*-values calculated using a chi^2 test for trend for IgG prevalence in different age groups at the same cross sectional bleed (*P*-values ≤ 0.05 indicated in bold type).

[¢] Missing values are due to a lack of children in that age group.

All samples included in analysis.

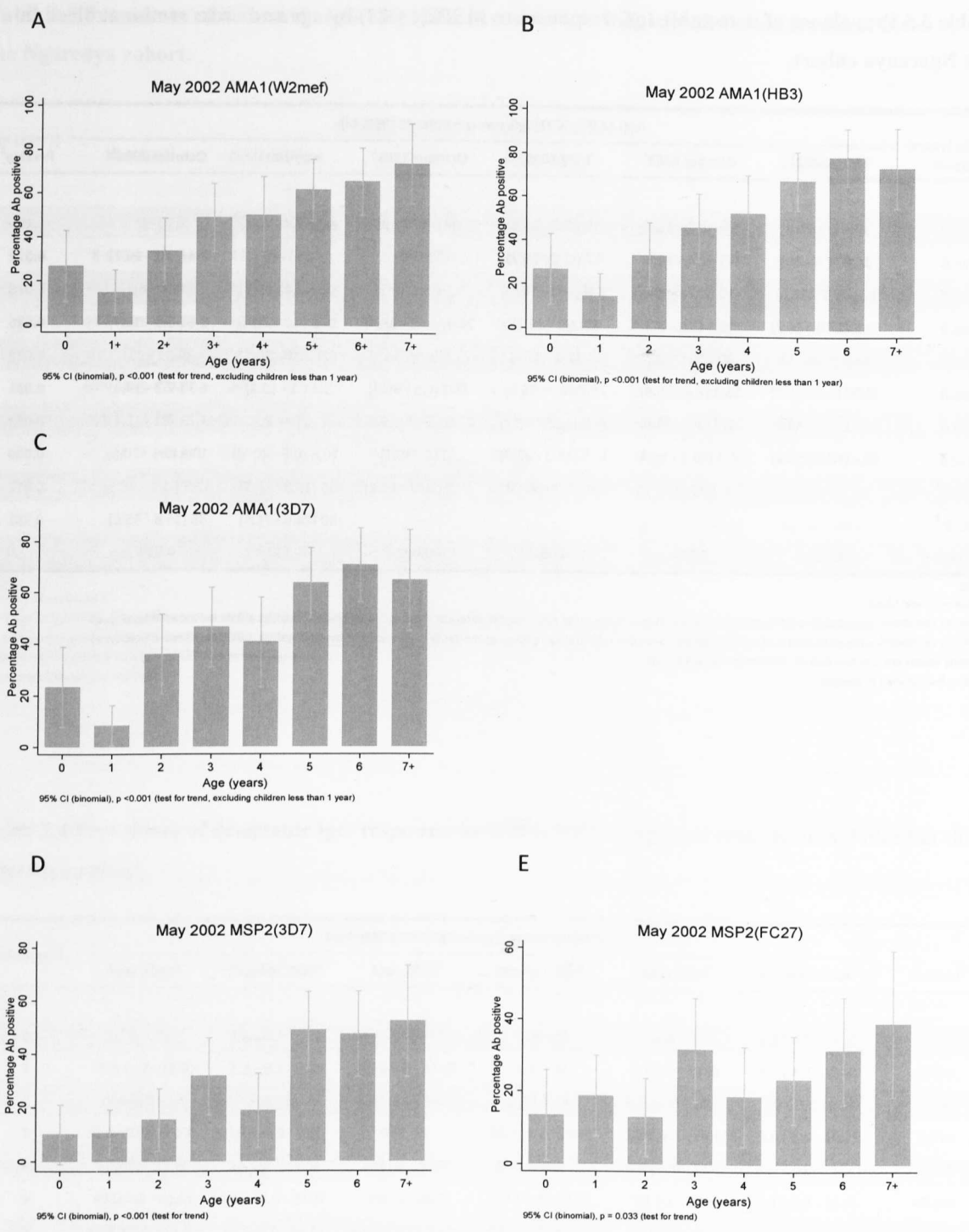

Figure 3.1 Proportion of children with detectable IgG responses to *P. falciparum* merozoite antigens by age group. Representative graphs showing prevalence at the May 2002 cross sectional bleed. All samples tested by ELISA. Error bars indicate 95% CI. Chi^2 test for trend excluding age 0 years for AMA1 variants. Chi^2 test for trend including age 0 years for MSP2 variants. (A) AMA1(W2mef) p <0.001, (B) AMA1(HB3) p <0.001, (C) AMA1(3D7) p <0.001, (D) MSP2(3D7) p <0.001, (E) MSP2(FC27) p = 0.033.

The magnitude of the antibody response to both AMA1 and MSP2 variants was analysed in four age groupings (children less than a year, 1-3 years, 4-6 years, and 7-10 years) at each cross sectional bleed. For all antigens at each cross sectional bleed, there was an increasing magnitude of response with increasing age ($p<0.001$, Kruskal-Wallis test) (Appendix 1 Tables 1-5). Analysis of actual OD values indicated that in all age groups there were a number of individuals who made very low responses to the various AMA1 and MSP2 variants, but the upper range of these responses tended to rise with increasing age (Figure 3.2, Table 3.6, Appendix 1 Tables 1-5). For all antigens tested there was a significant ($p <0.001$, Wilcoxon rank sum test) difference in the median IgG response between 1-3 year olds and 7-10 year olds (Table 3.6, Appendix 1 Tables 1-5). This was also true of the IgG response to A4 schizont extract which was used as a proxy of exposure (discussed later in this chapter). Conversely, the IgG response to a non-malaria antigen (tetanus toxoid) significantly declined with increasing age (Table 3.6), possibly because younger children would have received booster immunisations for tetanus over the study period.

3.3.2 Acquisition of IgG responses to AMA1 and MSP2 increase with measures of exposure

IgG responses to schizont protein extract were measured as a proxy for overall exposure to blood-stage *P. falciparum*. This measure was split into tertiles that indicated low, medium, and high levels of reactivity. The antibody response to AMA1 and MSP2 variants was then analysed with regards to these categories. Median IgG responses to all AMA1 variants increased with increasing exposure ($p \leq 0.05$, Kruskall Wallis test) at all cross-sectional bleeds (Figure 3.3) except those to AMA1(HB3) in May 2004 ($p = 0.70$) (Appendix 3 Tables 7-9). A similar increase in IgG responses to MSP2 with increasing exposure was seen, except in the response to MSP2(FC27) in May 2004 ($p = 0.208$) (Appendix 1 Tables 10-11). Significant differences in the median IgG response between categories ($p \leq 0.05$, Wilcoxon rank sum test) was observed for all antigens at each cross-sectional bleed except in those two cases mentioned above (Appendix 1 Tables 7-11).

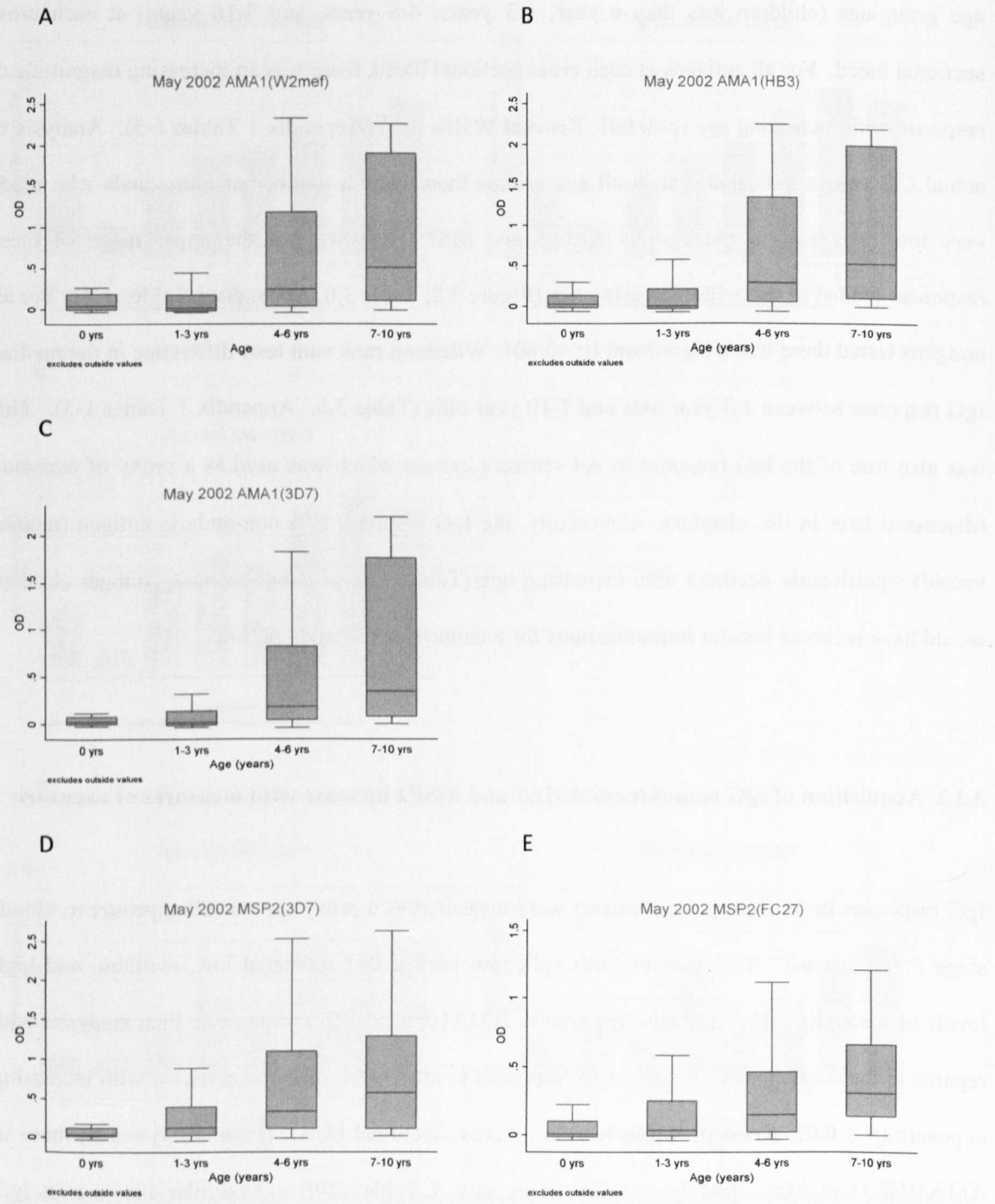

Figure 3.2 Magnitude of the IgG response to *P. falciparum* merozoite antigens by age group. Representative graphs showing IgG levels at the May 2002 cross sectional bleed. All samples tested by ELISA. Kruskal-Wallis test for equality of medians in all four age groups; $p < 0.001$ for all antigens. Wilcoxon rank sum test for equality of medians between children ages 1-3 years and 7-10 years; $p < 0.001$ for all antigens. (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7), (E) MSP2(FC27).

Table 3.6 IgG responses by age group and parasite status at the May 2002 cross sectional bleed.

May 2002 cross sectional bleed		All Samples					Aparasitaemic					Parasitaemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]
AMA1(W2mef)[1]	N[2]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.04	0.01	0.24	0.24	**<0.001***	0.04	-0.01	0.10	0.44	**<0.001***	0.00	0.65	0.89	1.30	0.710
	IQR	(-0.01 - 0.18)	(-0.02 - 0.17)	(0.03 - 1.18)	(0.1 - 1.9)		(-0.01 - 0.18)	(-0.03 - 0.09)	(0.02 - 0.59)	(0.13 - 1.67)		(0 - 0)	(0.11 - 1.67)	(0.43 - 2.02)	(0.06 - 2.19)	
AMA1(HB3)[1]	N[2]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.02	0.00	0.29	0.29	**<0.001***	0.02	-0.02	0.15	0.40	**<0.001***	0.00	0.49	0.91	1.40	0.471
	IQR	(-0.02 - 0.14)	(-0.04 - 0.22)	(0.05 - 1.34)	(0.1 - 1.98)		(-0.02 - 0.14)	(-0.04 - 0.07)	(0.04 - 0.77)	(0.18 - 1.78)		(0 - 0)	(0.12 - 1.87)	(0.39 - 1.99)	(0.09 - 2.17)	
AMA1(3D7)[1]	N[2]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.03	0.02	0.18	0.18	**<0.001***	0.03	0.00	0.11	0.32	**<0.001***	0.00	0.48	0.60	1.11	0.769
	IQR	(0 - 0.08)	(-0.01 - 0.14)	(0.04 - 0.82)	(0.07 - 1.77)		(0 - 0.08)	(-0.02 - 0.06)	(0.03 - 0.61)	(0.07 - 1.48)		(0 - 0)	(0.06 - 1.7)	(0.22 - 1.66)	(0.07 - 2.15)	
MSP2(3D7)[1]	N[2]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.04	0.13	0.33	0.33	**<0.001***	0.04	0.10	0.28	0.29	**<0.001***	0.00	0.89	1.14	1.76	0.640
	IQR	(0.02 - 0.13)	(0.03 - 0.4)	(0.12 - 1.09)	(0.11 - 1.29)		(0.02 - 0.13)	(0.03 - 0.3)	(0.12 - 0.82)	(0.11 - 0.87)		(0 - 0)	(0.58 - 1.58)	(0.16 - 2.08)	(0.33 - 2.43)	
MSP2(FC27)[1]	N[2]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.01	0.04	0.15	0.15	**<0.001***	0.01	0.02	0.10	0.30	**<0.001***	0.00	0.64	0.39	0.40	0.990
	IQR	(-0.01 - 0.1)	(0 - 0.25)	(0.02 - 0.47)	(0.13 - 0.67)		(-0.01 - 0.1)	(-0.01 - 0.18)	(0.01 - 0.36)	(0.11 - 0.61)		(0 - 0)	(0.18 - 1.37)	(0.2 - 1.79)	(0.22 - 0.94)	
Schizont Extract[1]	N[2]	30	127	117	23		30	108	93	15		0	19	24	8	
	Median	0.15	0.22	0.60	0.60	**<0.001***	0.15	0.17	0.46	0.64	**<0.001***	0.00	0.94	0.99	1.15	0.520
	IQR	(0.07 - 0.28)	(0.09 - 0.56)	(0.29 - 1.06)	(0.37 - 1.23)		(0.07 - 0.28)	(0.08 - 0.33)	(0.25 - 0.95)	(0.3 - 1.09)		(0 - 0)	(0.73 - 1.21)	(0.64 - 1.53)	(0.87 - 1.23)	
Tetanus Toxoid[1]	N[2]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.87	0.67	0.43	0.43	**<0.001***	0.87	0.68	0.46	0.45	**0.007**	0.00	0.64	0.38	0.23	**0.046***
	IQR	(0.44 - 1.43)	(0.33 - 1.04)	(0.28 - 0.73)	(0.2 - 0.58)		(0.44 - 1.43)	(0.35 - 1.02)	(0.29 - 0.77)	(0.27 - 0.62)		(0 - 0)	(0.28 - 1.1)	(0.2 - 0.64)	(0.14 - 0.42)	

Notes

[1] Antigens tested.

[2] Number of samples tested by ELISA.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

* *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

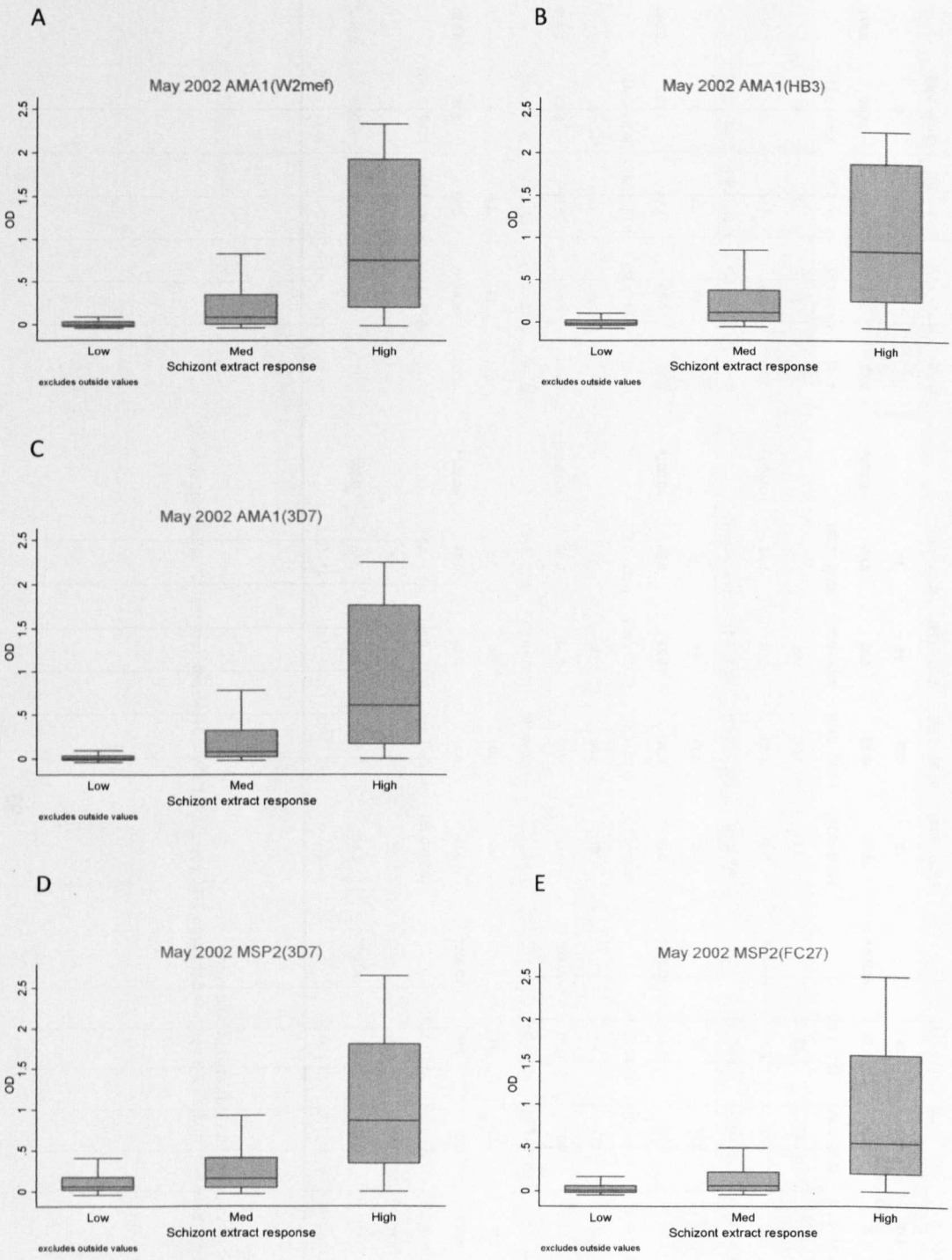

Figure 3.3 Magnitude of the IgG response to *P. falciparum* merozoite antigens by reactivity to A4 schizont extract protein. Representative graphs showing IgG levels at the May 2002 cross sectional bleed. All samples tested by ELISA. Kruskal-Wallis test for equality of median responses in all three categories; p <0.001 for all antigens. Wilcoxon rank sum test for equality of median responses between two categories; p <0.001 for all antigens. (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7), (E) MSP2(FC27).

3.3.3 Declining malaria transmission affects the acquisition of IgG responses to AMA1 and MSP2

Median IgG responses to all the AMA1 and MSP antigens tested, along with those to schizont extract for all samples were different at each cross sectional bleed ($p \leq 0.05$, Kruskal Wallis test) which could reflect differences in the malaria transmission levels at each time point (Table 3.7). As described in chapter 2, malaria transmission was on the decline over the duration of this study, particularly in the last year. Parasite prevalence fell from 17.1% in May 2002 to 3.7% in October 2004.

Table 3.7 Median IgG levels for each antigen over the study period

	IgG levels to merozoite antigens, median (IQR)					
	AMA1(W2mef)[2]	AMA1(HB3)[2]	AMA1(3D7)[2]	MSP2(3D7)[2]	MSP2(FC27)[2]	Schizont extract
May 2002[1] (308)	0.08 (0 - 0.59)	0.09 (-0.01 - 0.65)	0.07 (0 - 0.47)	0.2 (0.05 - 0.67)	0.1 (0.01 - 0.41)	0.37 (0.14 - 0.9)
October 2002[1] (294)	0.4 (0.1 - 1.37)	0.1 (0.01 - 0.44)	0.06 (0 - 0.29)	0.08 (0.03 - 0.21)	0.08 (0.02 - 0.32)	0.13 (0.05 - 0.44)
May 2003[1] (293)	0.05 (-0.01 - 0.49)	0.11 (0.01 - 0.61)	0.07 (0.01 - 0.48)	0.09 (0.03 - 0.27)	0.09 (0.03 - 0.28)	0.17 (0.05 - 0.53)
October 2003[1] (299)	0.07 (0.01 - 0.59)	0.05 (-0.04 - 0.57)	0.07 (0 - 0.43)	0.12 (0.04 - 0.35)	0.1 (0.02 - 0.37)	0.2 (0.07 - 0.71)
May 2004[1] (299)	0.04 (0 - 0.34)	0.05 (-0.01 - 0.37)	0.04 (-0.01 - 0.27)	0.07 (0.02 - 0.24)	0.07 (0.02 - 0.23)	0.09 (0.03 - 0.29)
October 2004[1] (302)	0.02 (-0.02 - 0.26)	0.01 (-0.05 - 0.24)	0.02 (-0.01 - 0.2)	0.07 (0.02 - 0.18)	0.05 (0.02 - 0.19)	0.08 (0.03 - 0.22)
***P*-value[3]**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.02**	**< 0.001**

Notes
[1] Cross sectional bleed, numbers in brackets indicate samples tested by ELISA.
[2] Recombinant merozoite antigens tested.
[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).
All samples tested

To examine whether antibody acquisition might be dependent on the levels of malaria transmission at the time that a serum sample was taken, sero-prevalence to all AMA1 and MSP2 variants was analysed in different age groups (0 to 9 years of age, in one year intervals) at different cross sectional bleeds and a test for trend carried out in each separate age group (Tables 3.1-3.5). At all cross sectional bleeds there was a significant rise ($p \leq 0.05$, chi^2 test for trend) in IgG prevalence as mentioned in an earlier section. Although there was a trend for reduced antibody prevalence in each age group over the study period this trend was only significant ($p \leq 0.05$, chi^2 test for trend) in 2-8 year olds for AMA1 variants (Tables 3.1-3.3), 3 year olds and 5-8 year olds for MSP2(3D7) (Table 3.4), and 1-8 year olds for MSP2(FC27) (Table 3.5). AMA1(HB3) (Table 3.2), 2- and 5-

year olds for AMA1(3D7) (Table 3.3), and 7-year olds for MSP2(FC27) (Table 5). Overall, a reduction in malaria transmission (inferred by a reduction in parasite prevalence at sample time) was accompanied by a reduction in antibody prevalence in all age groups.

3.3.4 Concurrent parasitaemia is associated with higher IgG responses to AMA1 and MSP2

For all the malaria antigens tested, children with concurrent parasitaemia at sampling had higher IgG responses than those in the same age group without concurrent parasitaemia. The IgG response to AMA1 and MSP2 variants and shizont extract at each cross sectional bleed increased significantly ($p < 0.001$, Kruskal Wallis test) with age in aparasitaemic children, with a significant difference between children ages 1-3 years and 7-10 years ($p \leq 0.05$, Wilcoxon rank sum test) (Table 3.6, Appendix 3 Tables 1-6). This was not the case for children with concurrent parasitaemia. The presence of parasites appeared to boost IgG responses in all children to the point that any age related increases were not seen (Table 3.6, Appendix 3 Tables 1-6). To show that the effect of parasites was specific to the malaria antigen response, examination of IgG responses to tetanus toxoid showed no differences in aparasitaemic or parasitaemic children (Table 3.6).

3.3.5 Diminished IgG responses are observed in the presence of both the sickle trait and alpha-thalassemia

Both the sickle trait and alpha-thalassemia are associated with protection from clinical malaria, particularly severe malaria. The IgG response to AMA1 and MSP2 variants was investigated with regards to the mentioned haemoglobinopathies by comparing these responses to those in normal children. IgG levels to AMA1 variants in children with the sickle trait (HbAS) were lower than in their normal (HbAA) counterparts (Figure 3.4, Table 3.8, Appendix 1 Tables 12-14), though this difference was significant only in a few cross sectional bleeds. When the entire cohort was analysed at each bleed, only in May 2003 were the median IgG responses to all three AMA1 variants significantly lower in HbAS children ($p \leq 0.05$, Wilcoxon rank test) (Appendix 1 Tables 12-14). IgG responses to the MSP2 variants in HbAS children had marginally reduced median

levels which were not significant at any of the cross-sectional bleeds (Figure 3.4, Table 3.8, Appendix 1 Tables 15-16).

The protection that is conferred by this alpha-thalassemia is only evident in homozygous individuals. IgG responses to AMA1 variants were similar in normal and heterozygous children (Figure 3.4, Table 3.8, Appendix 1 Tables 12-14) whereas those of the homozygotes were lower at four of the six cross sectional bleeds (October 2002 through to May 2004) ($p \leq 0.05$, Wilcoxon rank test) (Appendix 1 Tables 12-14). In contrast, though the IgG responses to MSP2 variants were consistently lower in range in homozygotes compared to heterozygotes and normal children (Figure 3.4, Table 3.8, Appendix 1 Tables 15-16), there was no difference in the median IgG responses between the three categories.

Reduced IgG responses to A4 schizont extract were seen in HbAS children only at the May 2002 cross sectional bleed ($p = 0.34$, Wilcoxon rank test) (Figure 3.5, Table 3.8, Appendix 1 Table 17). Though levels tended to be lower, there was no significant difference in the IgG response to A4 schizont extract by alpha-thalassemia homozygotes compared to the other two groups except at the October 2002 cross sectional bleed ($p \leq 0.05$, Wilcoxon rank test comparing median IgG levels in heterozygotes and homozygotes) (Appendix 1 Table 17). IgG responses to a non-malarial antigen were also measured in order to clarify that these antibody responses were specific to *P. falciparum*. There was no difference in antibody titres to tetanus toxoid in both HbAS and alpha-thalassemia homozygotes compared to their normal counterparts (Figure 3.5, Table 3.8, Appendix 1 Table 18) though there was an unusual significant increase in IgG responses to tetanus toxoid in homozygotes compared with heterozygotes ($p \leq 0.05$, Wilcoxon rank test) at the October 2003 cross sectional bleed (Appendix 1 Table 18). However it was not possible to account for tetanus immunisations that may have been given during the study which may be confounding these associations.

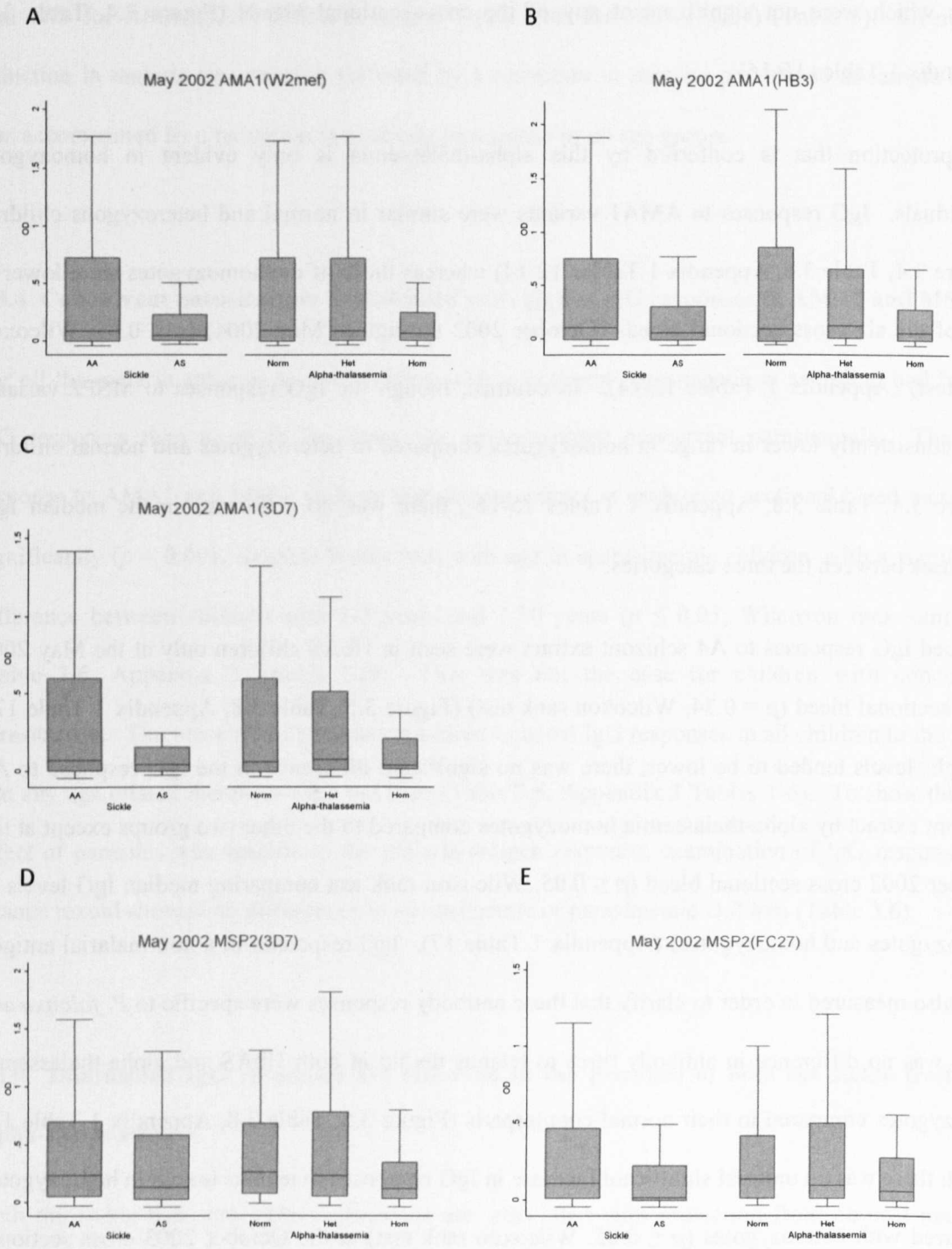

Figure 3.4 Magnitude of the antibody (IgG) response to *P. falciparum* merozoite antigens in the presence or absence of haemoglobinopathies (sickle trait and α-thalassemia). AA=Normal, AS=Sickle trait, Norm=Normal, Het=Heterozygous, Hom=Homozygous. Representative graphs showing IgG levels at the May 2002 cross sectional bleed. All samples tested by ELISA. Kruskal-Wallis test for equality of median responses in α-thalassemia categories non-significant for all antigens. Wilcoxon rank sum test for equality of median responses between two sickle trait categories non-significant for all antigens except MSP2(FC27). (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7): $p = 0.059$, Kruskal-wallis test on α-thalassemia medians, (E) MSP2(FC27): $p = 0.052$, Wilcoxon rank sum test on HbAA and HbAS medians.

Table 3.8 IgG responses in the presence or absence of haemoglobinopathies at the May 2002 cross sectional bleed.

May 2002[Δ]		Sickle trait			Alpha-Thalassemia			
		HbAA	HBAS	*P*-value[3]	Norm	Het	Homo	*P*-value[4]
AMA1(W2mef)[1]	N[2]	258	41		93	152	52	
	Median	0.09	0.05	0.215	0.09	0.09	0.07	0.521
	IQR	(-0.01 - 0.72)	(0 - 0.23)		(-0.01 - 0.72)	(0 - 0.72)	(0 - 0.25)	
AMA1(HB3)[1]	N[2]	258	41		93	152	52	
	Median	0.10	0.05	0.258	0.09	0.12	0.05	0.169
	IQR	(-0.01 - 0.75)	(-0.02 - 0.3)		(-0.03 - 0.85)	(0 - 0.65)	(-0.02 - 0.27)	
AMA1(3D7)[1]	N[2]	258	41		93	152	52	
	Median	0.07	0.04	0.162	0.06	0.08	0.04	0.357
	IQR	(0 - 0.6)	(0 - 0.15)		(-0.01 - 0.59)	(0.01 - 0.51)	(0.01 - 0.21)	
MSP2(3D7)[1]	N[2]	258	41		93	152	52	
	Median	0.24	0.13	0.141	0.26	0.23	0.12	0.059
	IQR	(0.05 - 0.69)	(0.02 - 0.58)		(0.07 - 0.68)	(0.05 - 0.78)	(0.03 - 0.34)	
MSP2(FC27)[1]	N[2]	258	41		93	152	52	
	Median	0.11	0.01	0.052	0.14	0.09	0.06	0.397
	IQR	(0.01 - 0.47)	(0 - 0.22)		(0.01 - 0.42)	(0.01 - 0.5)	(-0.01 - 0.28)	
Schizont extract[1]	N[2]	257	41		92	153	51	
	Median	0.41	0.25	**0.034**	0.43	0.41	0.33	0.273
	IQR	(0.16 - 0.92)	(0.08 - 0.72)		(0.15 - 0.95)	(0.16 - 0.91)	(0.09 - 0.68)	

Notes

[1] Antigens tested.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Wilcoxon rank test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[Δ] Cross-sectional sampling time.

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children heterozygous for α-thalassemia (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children homozygous for α-thalassemia (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children heterozygous and homozygous for α-thalassemia (*P*-values not shown).

Possible effects of sickle trait and alpha-thalassemia on the acquisition of antibodies was also investigated. Samples were grouped according to the age of the individual at sample time (less than one year, 1-3 years, 4-6 years, and 7-10 years). IgG responses to AMA1 and MSP2 were then analysed.

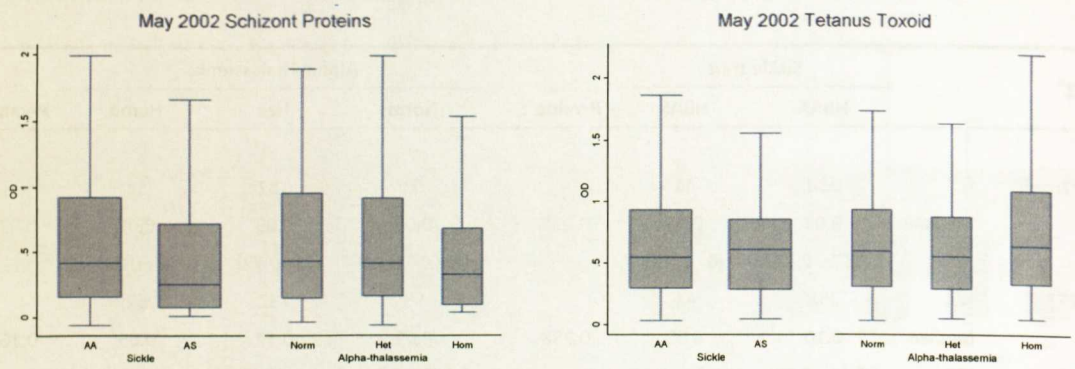

Figure 3.5 Magnitude of the antibody (IgG) response to *P. falciparum* merozoite antigens in the presence or absence of haemoglobinopathies (sickle trait and α-thalassemia). AA=Normal, AS=Sickle trait, Norm=Normal, Het=Heterozygous, Hom=Homozygous. Representative graphs showing IgG levels at the May 2002 cross sectional bleed. All samples tested by ELISA. Kruskal-Wallis test for equality of median responses in α-thalassemia categories non-significant for all antigens. (A) A4 schizont extract: p = 0.034, Wilcoxon rank sum test on HbAA and HbAS medians, (B) tetanus toxoid.

For all AMA1 and MSP2 variants, there was a significant increase in antibody titres in normal (HbAA) children with increasing age at all cross-sectional bleeds (p <0.001, Kruskal Wallis test; $p \leq 0.05$, Wilcoxon rank test comparing median levels between children ages 1-3 years and 7-10 years) (Figure 3.6, Table 3.9, Appendix 1 Tables 19-23). These levels were in contrast to HbAS children who did not appear to exhibit the same age related acquisition of IgG to the antigens tested at most cross sectional bleeds. Only in a few cases was there a significant association of antibody responses with age. HbAs children ages 7-10 had significantly higher IgG responses to AMA1(W2mef) compared to 1-3 year olds in May 2003 ($p \leq 0.05$, Wilcoxon rank test) (Appendix 1 Table 19). There was an age related increase in IgG responses to MSP2(3D7) and MSP(FC27) in October 2003 and May 2002 respectively ($p \leq 0.05$, Kruskal Wallis test) (Figure 3.6, Table 3.9, Appendix 1 Tables 22-23).

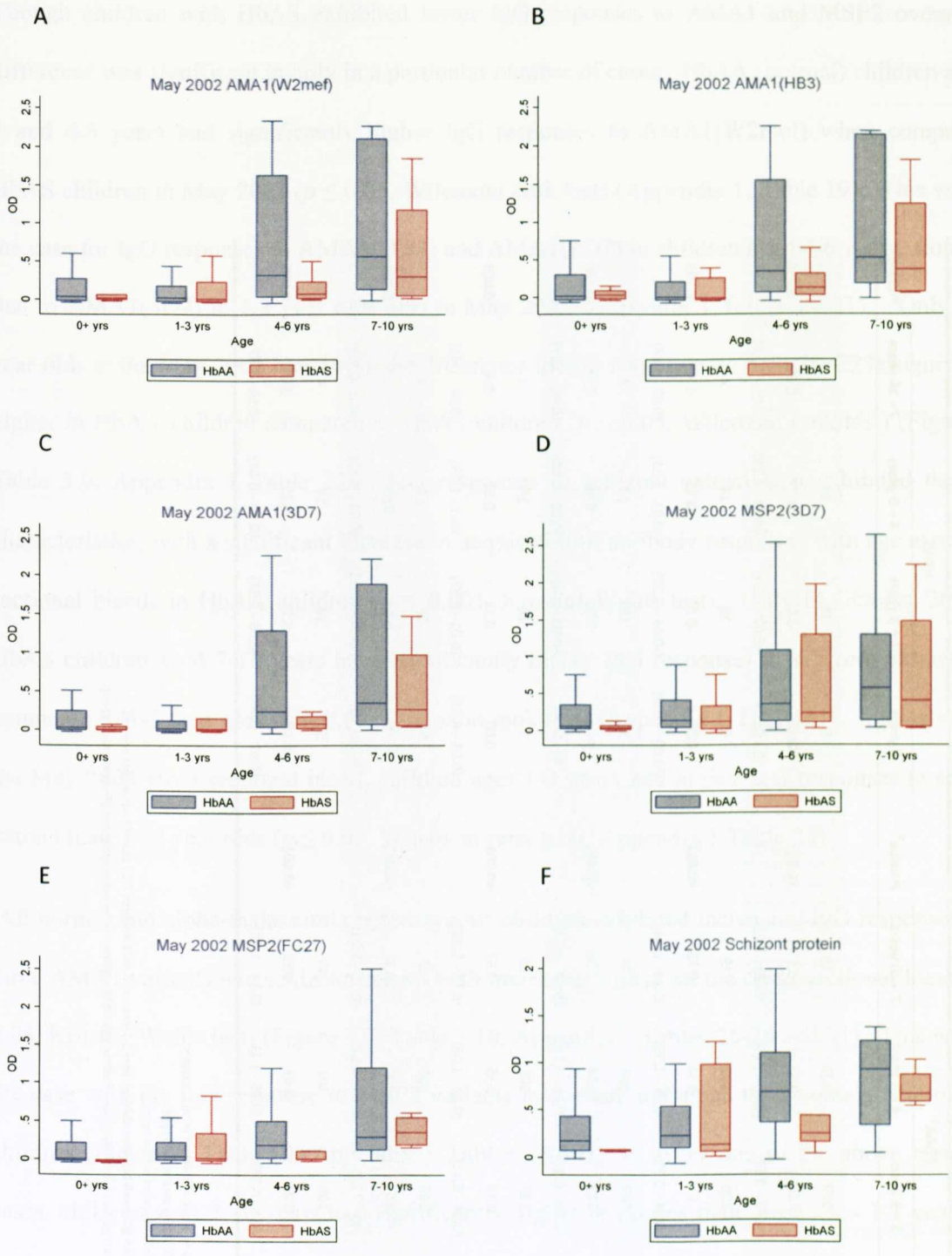

Figure 3.6 Magnitude of the antibody (IgG) response to *P. falciparum* merozoite and schizont antigens in the presence and absence of sickle trait by age group. HbAA = Normal, HbAS = Sickle trait. Representative graphs showing IgG levels at the May 2002 cross sectional bleed. All samples tested by ELISA. Kruskal-Wallis test for equality of median responses in HbAA: $p < 0.001$ for all antigens. Wilcoxon rank sum test for equality of median responses between children aged 1-3 years and 7-10 years in HbAA: $p \leq 0.05$ for all antigens. No significant associations for HbAS. Detail of further tests in Table 3.9. (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7), (E) MSP2(FC27), (F) A4 Schizont proteins.

Table 3.9 IgG responses in the presence and absence of the sickle trait at the May 2002 cross sectional bleed.

May 2002		HbAA[Δ]					HbAS[Δ]				
		0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[3]	0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[4]
AMA1(W2mef)[1]	N[2]	23	109	102	19		5	18	14	4	
	Median	0.04	0.02	0.32	0.57	**<0.001**[*]	0.02	0.01	0.07	0.29	0.258
	IQR	(-0.01 - 0.26)	(-0.02 - 0.17)	(0.03 - 1.63)	(0.13 - 2.1)		(0 - 0.06)	(-0.02 - 0.23)	(0.01 - 0.23)	(0.05 - 1.18)	
AMA1(HB3)[1]	N[2]	23	109	102	19		5	18	14	4	
	Median	0.01	0	0.34	0.53	**<0.001**[*]	0.03	-0.01	0.12	0.36	0.135
	IQR	(-0.03 - 0.29)	(-0.04 - 0.21)	(0.06 - 1.55)	(0.18 - 2.14)		(-0.02 - 0.08)	(-0.03 - 0.25)	(0.03 - 0.31)	(0.07 - 1.24)	
AMA1(3D7)[1]	N[2]	23	109	102	19		5	18	14	4	
	Median	0.03	0.02	0.24	0.35	**<0.001**[*]	0.01	0.01	0.08	0.27	0.104
	IQR	(0 - 0.25)	(-0.01 - 0.12)	(0.04 - 1.28)	(0.07 - 1.88)		(0 - 0.04)	(-0.01 - 0.15)	(0.02 - 0.18)	(0.06 - 0.97)	
MSP2(3D7)[1]	N[2]	23	109	102	19		5	18	14	4	
	Median	0.05	0.14	0.35	0.57	**<0.001**[*]	0.03	0.12	0.23	0.4	0.069
	IQR	(0.02 - 0.34)	(0.04 - 0.41)	(0.12 - 1.08)	(0.13 - 1.29)		(0.01 - 0.06)	(0.01 - 0.33)	(0.1 - 1.29)	(0.04 - 1.48)	
MSP2(FC27)[1]	N[2]	23	109	102	19		5	18	14	4	
	Median	0.01	0.04	0.2[†]	0.31	**<0.001**[*]	-0.01	0.02	0.01[†]	0.37	**0.049**
	IQR	(-0.01 - 0.22)	(0 - 0.23)	(0.03 - 0.51)	(0.13 - 1.21)		(-0.02 - 0)	(-0.01 - 0.35)	(0.01 - 0.12)	(0.2 - 0.55)	
Schizont extract[1]	N[2]	23	108	102	19		5	18	14	4	
	Median	0.18	0.23	0.69[†]	0.93	**<0.001**[*]	0.08	0.14	0.25[†]	0.68	0.095
	IQR	(0.07 - 0.43)	(0.1 - 0.54)	(0.36 - 1.1)	(0.34 - 1.23)		(0.07 - 0.08)	(0.06 - 0.98)	(0.17 - 0.44)	(0.59 - 0.87)	

Notes

[1] Antigens tested.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between HbAA children and HbAS children in the same age group (*P*-values not shown).

[Δ] HbAA = normal, HbAS = Sickle trait

Though children with HbAS exhibited lower IgG responses to AMA1 and MSP2 overall, this difference was significant in only in a particular number of cases. HbAA (normal) children aged 1-3 and 4-6 years had significantly higher IgG responses to AMA1(W2mef) when compared to HbAS children in May 2003 ($p \leq 0.05$, Wilcoxon rank test) (Appendix 1, Table 19). This was also the case for IgG responses to AMA1(HB3) and AMA1(3D7) in children aged 4-6 years, along with that to AMA1(3D7) in 1-3 year olds also in May 2003 (Appendix 1 Tables 20-21). Only in 4-6 year olds at the May 2002 bleed was the difference in IgG responses to MSP2(FC27) significantly higher in HbAA children compared to HbAS children ($p \leq 0.05$, Wilcoxon rank test) (Figure 3.6, Table 3.9, Appendix 1 Table 23). IgG responses to schizont extract also exhibited the same characteristics, with a significant increase in acquisition of antibody responses with age at all cross sectional bleeds in HbAA children ($p < 0.001$, Kruskal Wallis test). Only in October 2004 did HbAS children aged 7-10 years have significantly higher IgG responses to schizont extract when compared to 1-3 year olds ($p \leq 0.05$, Wilcoxon rank test) (Appendix 1 Table 24). Interestingly, at the May 2004 cross sectional bleed, children ages 1-3 years had *higher* IgG responses to schizont extract than 7-10 year olds ($p \leq 0.05$, Wilcoxon rank test) (Appendix 1 Table 24).

All normal and alpha-thalassemia heterozygous children exhibited increasing IgG responses to all three AMA1 variants and schizont extract with increasing age at all the cross-sectional bleeds ($p \leq 0.05$, Kruskal Wallis test) (Figure 3.7, Table 3.10, Appendix 1 Tables 26-28 and 31). This was also the case with the IgG response to MSP2 variants in normal and alpha-thalassemaia heterozygous children (Figure 7, Table 10, Appendix 1 Tables 29-30). In every one of the above mentioned cases, children ages 7-10 years had significantly higher responses than those ages 1-3 years ($p \leq 0.05$, Wilcoxon rank test) (Figure 3.7, Table 3.10, Appendix 1 Tables 26-31). Children who were alpha-thalassemia homozygous also exhibited an age dependent acquisition of IgG responses to all AMA1 variants and schizont extract at most of the cross sectional bleeds though it was not always accompanied by a significant difference in levels between 7-10 year olds and 1-3 year olds due to small numbers in each category (Figure 3.7, Table 3.10, Appendix 1 Tables 26-28 and 31). Unlike the case for AMA1, alpha-thalassemia homozygotes did not exhibit a significant age related acquisition of IgG responses to MSP2 variants except in May 2002 ($p = 0.009$, Kruskal Wallis test) and May 2004 ($p \leq 0.05$, Wilcoxon rank test comparing median levels between children ages 1-3

years and 7-10 years) for MSP2(3D7) (Figure 3.7, Table 3.10, Appendix 1 Table 29) and October 2002 ($p = 0.018$, Kruskal Wallis test) and both May 2003 and October 2003 ($p \leq 0.05$, Wilcoxon rank test comparing median levels between children ages 1-3 years and 7-10 years) for MSP2(FC27) (Appendix 1 Table 30).

3.3.6 Age, concurrent parasitaemia, haemoglobinopathies, and prior *P. falciparum* exposure have variable effects on IgG responses which are dependent on malaria transmission levels

Previous sections of this chapter have highlighted the different factors that affect antibody levels. Age, concurrent parasitaemia, erythrocyte polymorphisms, and prior exposure have different effects on the antibody outcome. In order to investigate this further I carried out a series of univariate and multivariable linear regression analyses on the antibody levels to all AMA1 and MSP2 variants at different samples times to try to quantify the effect of different variables on IgG responses in this cohort. IgG levels to each antigen were analysed at separate time points rather than pooling them as malaria transmission dropped over the study period as it was thought that this could confound the results.

Age in years, parasite status at sample time, alpha-thalassemia status, sickle trait, and recorded episodes of parasitaemia and clinical malaria in the prior six months were fitted into univariate linear regression models to determine their inclusion into a multivariable linear regression model. All the dependent variables were fitted as categorical variables except age which was a continuous variable, and parasite status which was fitted as a binary variable.

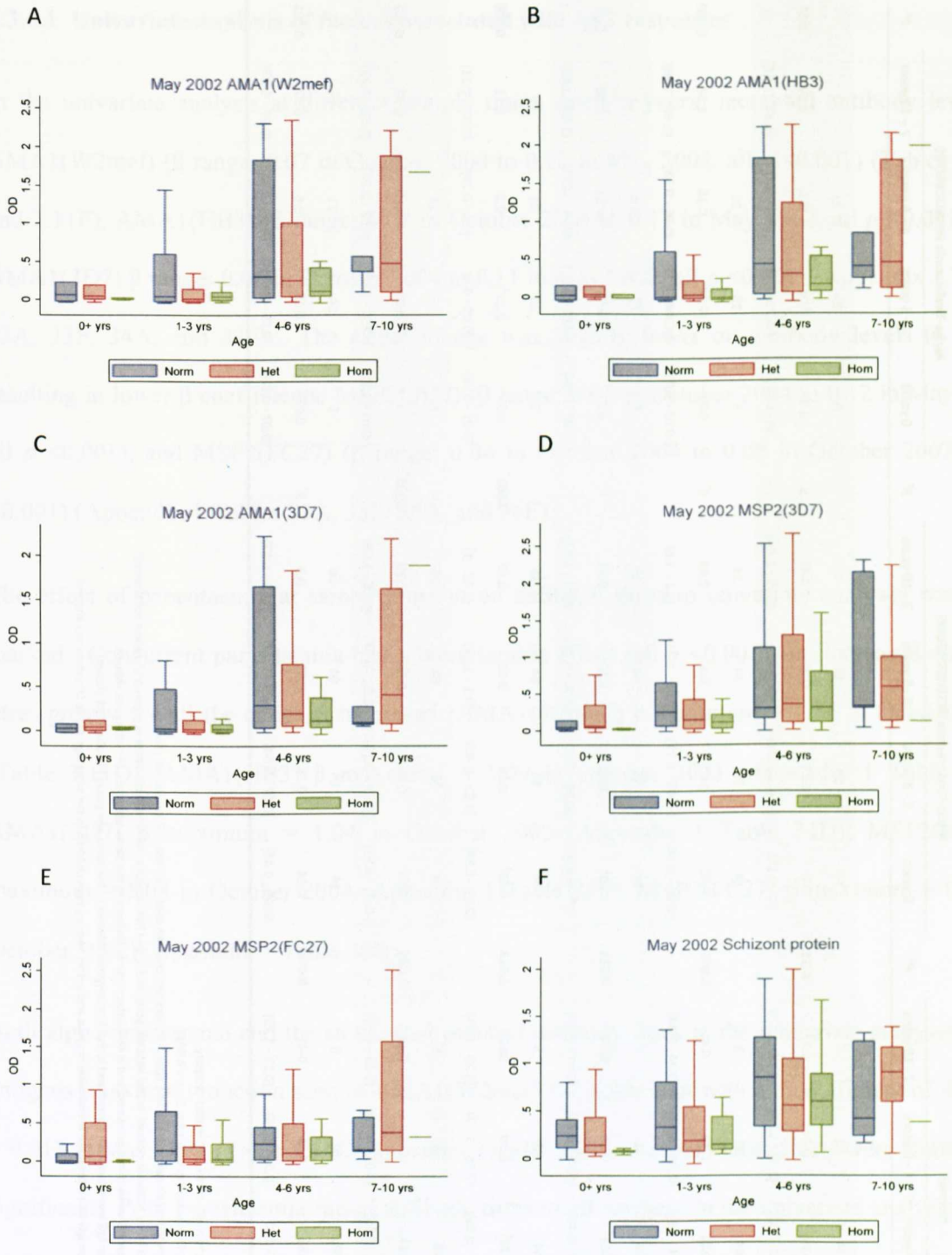

Figure 3.7 Magnitude of the antibody (IgG) response to *P. falciparum* merozoite and schizont antigens in the presence and absence of alpha-thalassemia by age group. Norm = Normal, Het = Heterozygous, Hom = Homozygous. Representative graphs showing IgG levels at the May 2002 cross sectional bleed. All samples tested by ELISA. Kruskal-Wallis test for equality of median responses in normal, heterozygotes and homozygotes by age: $p \leq 0.05$ for all antigens except the MSP2(FC27) response in homozygotes. Detail of further tests in Table 10. (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7), (E) MSP2(FC27), (F) schizont proteins.

Table 3.10 IgG responses in the presence and absence of alpha-thalassemia at the May 2002 cross sectional bleed.

May 2002		Normal					Alpha-Thalassemia HETEROZYGOUS					Alpha-Thalassemia HOMOZYGOUS				
		0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 – 3 years	4 – 6 years	7 – 10 years	P^3
AMA1(w2mef)[1]	N[2]	10	44	32	5		14	55	64	16		5	25	21	1	
	Median	0.06	0.04	0.3	0.37	**0.01**	0.04	0.01	0.25	0.47	S*	0.02	0.04	0.1	1.67	**0.03**
	IQR	(-0.02 - 0.23)	(-0.03 - 0.6)	(0.02 - 1.83)	(0.36 - 0.57)		(0 - 0.18)	(-0.02 - 0.13)	(0.04 - 1)	(0.09 - 1.88)		(0 - 0.02)	(-0.01 - 0.1)	(0.04 - 0.42)	(1.67 - 1.67)	
AMA1(HB3)[1]	N[2]	10	44	32	5		14	55	64	16		5	25	21	1	
	Median	0.01	-0.01	0.43	0.4	**0.001***	0.02	0.01	0.3	0.45	S*	0.03	-0.02	0.16	1.98	**0.003**
	IQR	(-0.05 - 0.14)	(-0.04 - 0.6)	(0.03 - 1.82)	(0.24 - 0.84)		(-0.01 - 0.14)	(-0.03 - 0.21)	(0.05 - 1.24)	(0.1 - 1.8)		(-0.02 - 0.03)	(-0.04 - 0.1)	(0.06 - 0.53)	(1.98 - 1.98)	
AMA1(3D7)[1]	N[2]	10	44	32	5		14	55	64	16		5	25	21	1	
	Median	0.02	0.02	0.29	0.09	**0.011**	0.04	0.02	0.19	0.41	S*	0.03	0.02	0.11	1.88	**0.01**
	IQR	(-0.02 - 0.08)	(-0.01 - 0.47)	(0.03 - 1.65)	(0.07 - 0.27)		(0 - 0.12)	(-0.01 - 0.12)	(0.05 - 0.76)	(0.07 - 1.62)		(0.01 - 0.04)	(-0.01 - 0.07)	(0.03 - 0.37)	(1.88 - 1.88)	
MSP2(3D7)[1]	N[2]	10	44	32	5		14	55	64	16		5	25	21	1	
	Median	0.04	0.24[ε]	0.29	0.32	**0.013**	0.06[Σ]	0.14	0.35	0.59	**0.001***	0.03[Σ]	0.11[ε]	0.3	0.11	**0.009**
	IQR	(0.02 - 0.13)	(0.06 - 0.65)	(0.16 - 1.12)	(0.29 - 2.13)		(0.03 - 0.34)	(0.02 - 0.42)	(0.1 - 1.29)	(0.12 - 1)		(0.01 - 0.03)	(0.03 - 0.2)	(0.1 - 0.79)	(0.11 - 0.11)	
MSP2(FC27)[1]	N[2]	10	44	32	5		14	55	64	16		5	25	21	1	
	Median	0.01	0.14	0.23	0.16	**0.026**	0.07	0.03	0.12	0.38	**0.002***	0	0.03	0.12	0.32	0.162
	IQR	(-0.02 - 0.1)	(0.01 - 0.65)	(0.03 - 0.45)	(0.13 - 0.58)		(-0.01 - 0.51)	(-0.01 - 0.19)	(0.02 - 0.5)	(0.15 - 1.58)		(-0.01 - 0.01)	(-0.01 - 0.23)	(0.02 - 0.32)	(0.32 - 0.32)	
Schizont extract[1]	N[2]	10	43	32	5		14	56	64	16		5	24	21	1	
	Median	0.23[ε]	0.32	0.85	0.41	**0.014**	0.14	0.18	0.55	0.9	S*	0.07[ε]	0.15	0.6	0.37	**0.001**
	IQR	(0.11 - 0.41)	(0.1 - 0.8)	(0.32 - 1.28)	(0.23 - 1.23)		(0.06 - 0.43)	(0.07 - 0.54)	(0.27 - 1.04)	(0.59 - 1.17)		(0.07 - 0.08)	(0.09 - 0.43)	(0.34 - 0.88)	(0.37 - 0.37)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type, S = $P < 0.001$).

* Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

† Significant difference ($P \leq 0.05$ Kruskal Wallis) in median IgG levels between normal, α-thal heterozygous, and α-thal homozygous children in the same age group (*P*-values not shown).

‡ Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal and α-thal heterozygous children in the same age group (*P*-values not shown).

ε Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal and α-thal homozygous children in the same age group (*P*-values not shown).

Σ Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between α-thal heterozygous and α-thal homozygous children in the same age group (*P*-values not shown).

3.3.6.1 Univariate analysis of factors associated with IgG responses

In the univariate analysis at different sample times, age (in years) increased antibody levels to AMA1(W2mef) (β range: 0.07 in October 2004 to 0.12 in May 2002, all p <0.001) (Tables 3.11A and 3.11F), AMA1(HB3) (β range: 0.07 in October 2004 to 0.13 in May 2002, all p <0.001), and AMA1(3D7) β range: 0.06 in October 2004 to 0.11 in May 2002, all p <0.001) (Appendix 1 Tables 33A, 33F, 34A, and 34F). The effect of age was slightly lower on antibody levels to MSP2 resulting in lower β coefficients; MSP2(3D7) (β range: 0.05 in October 2004 to 0.12 in May 2002, all p <0.001), and MSP2(FC27) (β range: 0.04 in October 2004 to 0.08 in October 2003, all p <0.001) (Appendix 1 Tables 35A, 35F, 36A, and 36F).

The effect of parasitaemia at sample time in on antibody titres in univariate analyses was quite marked. Concurrent parasitaemia had a considerable effect (all p <0.001) and increased antibody titres greatly for all the antigens measured (AMA1(W2mef) β maximum = 1.14 in October 2003 (Table 3.11D); AMA1(HB3) β maximum = 1.09 in October 2003 (Appendix 1 Table 33D); AMA1(3D7) β maximum = 1.04 in October 2003 (Appendix 1 Table 34D); MSP2(3D7) β maximum = 1.08 in October 2004 (Appendix 1 Table 35F); MSP2(FC27) β maximum = 0.84 in October 2002) (Appendix 1 Table 36B).

Both alpha-thalassemia and the sickle trait reduced antibody titres in the univariate analyses to all antigens (maximal reduction seen in AMA1(W2mef) for sickle trait with a β coefficient of -0.32, p = 0.013 in May 2003 (Table 3.11C, Appendix 1), although not all of these effects were statistically significant. Prior parasitaemia raised antibody titres to all antigens in the univariate analyses (all p <0.001) with a maximum β coefficient of 0.32 for AMA1(HB3) in May 2003 (Appendix 1 Table 33C), with prior episodes of mild malaria also increasing (all p <0.001) antibody titres to all antigens with a maximum β coefficient of 0.36 for AMA1(HB3) in May 2002 (Appendix 1 Table 33A).

Table 3.11A Effect of different factors on IgG responses to AMA1(W2mef) in the May 2002 cross sectional bleed.

	AMA1(W2mef) May 2002[1]		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.12 (0.09-0.16)	**<0.001**	298
Parasitaemia	0.73 (0.53-0.93)	**<0.001**	298
Alpha-thal HET	-0.06 (-0.24-0.13)	0.549	297
Alpha-thal HOM	-0.29 (-0.53–0.04)	**0.02**	
Sickle trait	-0.23 (-0.47-0)	0.054	299
Prior parasitaemia	0.31 (0.22-0.39)	**<0.001**	304
Prior episode	0.34 (0.19-0.49)	**<0.001**	304
Multivariate (previous parasitaemia)[6]			
All samples			
Age (years)	0.08 (0.05-0.12)	**<0.001**	289
Parasitaemia	0.48 (0.28-0.68)	**<0.001**	
Alpha-thal HET	-0.04 (-0.2-0.12)	0.625	
Alpha-thal HOM	-0.18 (-0.39-0.03)	0.099	
Sickle trait	-0.2 (-0.41-0)	0.053	
Prior parasitaemia	0.19 (0.11-0.28)	**<0.001**	
Parasite negative[7]			
Age (years)	0.07 (0.04-0.1)	**<0.001**	239
Alpha-thal HET	-0.03 (-0.19-0.13)	0.745	
Alpha-thal HOM	-0.17 (-0.37-0.03)	0.093	
Sickle trait	-0.12 (-0.33-0.09)	0.253	
Prior parasitaemia	0.28 (0.19-0.37)	**<0.001**	
Parasite positive[8]			
Age (years)	0.12 (-0.03-0.27)	0.124	50
Alpha-thal HET	-0.29 (-0.88-0.3)	0.325	
Alpha-thal HOM	-0.39 (-1.38-0.59)	0.424	
Sickle trait	-0.44 (-1.09-0.21)	0.177	
Prior parasitaemia	-0.04 (-0.32-0.23)	0.742	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.1 (0.06-0.13)	**<0.001**	289
Parasitaemia	0.52 (0.31-0.72)	**<0.001**	
Alpha-thal HET	-0.06 (-0.23-0.1)	0.457	
Alpha-thal HOM	-0.18 (-0.4-0.03)	0.094	
Sickle trait	-0.2 (-0.41-0.01)	0.061	
Prior episode	0.21 (0.06-0.35)	**0.006**	
Parasite negative[7]			
Age (years)	0.09 (0.06-0.13)	**<0.001**	239
Alpha-thal HET	-0.04 (-0.21-0.13)	0.642	
Alpha-thal HOM	-0.18 (-0.39-0.03)	0.097	
Sickle trait	-0.15 (-0.37-0.06)	0.165	
Prior episode	0.27 (0.11-0.43)	**0.001**	
Parasite positive[8]			
Age (years)	0.13 (-0.03-0.28)	0.11	50
Alpha-thal HET	-0.26 (-0.83-0.32)	0.374	
Alpha-thal HOM	-0.37 (-1.39-0.64)	0.463	
Sickle trait	-0.44 (-1.11-0.23)	0.191	
Prior episode	0.01 (-0.41-0.43)	0.954	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitaemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 3.11B Effect of different factors on IgG responses to AMA1(W2mef) in the October 2002 cross sectional bleed.

	AMA1(W2mef) Oct 2002		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.12 (0.08-0.16)	**<0.001**	291
Parasitaemia	0.51 (0.12-0.89)	**0.011**	291
Alpha-thal HET	0.06 (-0.17-0.28)	0.63	284
Alpha-thal HOM	-0.26 (-0.55-0.02)	0.071	
Sickle trait	-0.27 (-0.58-0.03)	0.081	292
Prior parasitaemia	0.28 (0.09-0.47)	**0.004**	254
Prior episode	0.38 (0.13-0.62)	**0.003**	254
Multivariate (previous parasitaemia)[6]			
All samples			
Age (years)	0.19 (0.15-0.23)	**<0.001**	251
Parasitaemia	0.41 (0.07-0.76)	**0.018**	
Alpha-thal HET	0.01 (-0.2-0.22)	0.915	
Alpha-thal HOM	-0.19 (-0.45-0.07)	0.158	
Sickle trait	-0.26 (-0.54-0.02)	0.07	
Prior parasitaemia	0.23 (0.07-0.4)	**0.006**	
Parasite negative[7]			
Age (years)	0.2 (0.15-0.24)	**<0.001**	232
Alpha-thal HET	0.05 (-0.16-0.26)	0.64	
Alpha-thal HOM	-0.12 (-0.39-0.14)	0.353	
Sickle trait	-0.24 (-0.53-0.04)	0.093	
Prior parasitaemia	0.25 (0.08-0.42)	**0.003**	
Parasite positive[8]			
Age (years)	0.09 (-0.28-0.46)	0.602	19
Alpha-thal HET	0 (-1.58-1.57)	0.996	
Alpha-thal HOM	-1.02 (-2.94-0.9)	0.272	
Sickle trait	-0.62 (-2.34-1.1)	0.449	
Prior parasitaemia	-0.16 (-1.18-0.87)	0.745	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.19 (0.14-0.23)	**<0.001**	251
Parasitaemia	0.44 (0.09-0.78)	**0.013**	
Alpha-thal HET	0 (-0.2-0.21)	0.972	
Alpha-thal HOM	-0.19 (-0.45-0.07)	0.151	
Sickle trait	-0.28 (-0.56-0.01)	0.055	
Prior episode	0.3 (0.08-0.51)	**0.007**	
Parasite negative[7]			
Age (years)	0.19 (0.15-0.24)	**<0.001**	232
Alpha-thal HET	0.03 (-0.18-0.24)	0.757	
Alpha-thal HOM	-0.13 (-0.39-0.13)	0.327	
Sickle trait	-0.26 (-0.54-0.03)	0.075	
Prior episode	0.31 (0.1-0.53)	**0.005**	
Parasite positive[8]			
Age (years)	0.11 (-0.23-0.46)	0.491	19
Alpha-thal HET	-0.1 (-1.56-1.36)	0.887	
Alpha-thal HOM	-1.02 (-2.95-0.91)	0.274	
Sickle trait	-0.57 (-2.26-1.13)	0.482	
Prior episode	-0.02 (-1.11-1.08)	0.974	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitaemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 3.11C Effect of different factors on IgG responses to AMA1(W2mef) in the May 2003 cross sectional bleed.

	AMA1(W2mef) May 2003		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.11 (0.08-0.14)	**<0.001**	285
Parasitaemia	0.54 (0.3-0.78)	**<0.001**	285
Alpha-thal HET	0.05 (-0.14-0.24)	0.6	279
Alpha-thal HOM	-0.14 (-0.39-0.11)	0.272	
Sickle trait	-0.32 (-0.57--0.07)	**0.013**	283
Prior parasitaemia	0.07 (0-0.14)	0.053	253
Prior episode	0.13 (0.03-0.22)	**0.009**	253
Multivariate (previous parasitaemia)[6]			
All samples			
Age (years)	0.1 (0.06-0.14)	**<0.001**	247
Parasitaemia	0.41 (0.16-0.66)	**0.002**	
Alpha-thal HET	0.04 (-0.16-0.23)	0.717	
Alpha-thal HOM	-0.05 (-0.3-0.2)	0.687	
Sickle trait	-0.3 (-0.56--0.04)	**0.024**	
Prior parasitaemia	0.05 (-0.02-0.11)	0.177	
Parasite negative[7]			
Age (years)	0.09 (0.05-0.13)	**<0.001**	213
Alpha-thal HET	0.11 (-0.1-0.31)	0.297	
Alpha-thal HOM	-0.04 (-0.29-0.21)	0.753	
Sickle trait	-0.21 (-0.47-0.05)	0.118	
Prior parasitaemia	0.07 (0.01-0.14)	**0.031**	
Parasite positive[8]			
Age (years)	0.15 (0.02-0.29)	**0.026**	34
Alpha-thal HET	-0.21 (-0.84-0.42)	0.499	
Alpha-thal HOM	0.55 (-0.61-1.71)	0.343	
Sickle trait	-0.94 (-2.06-0.17)	0.094	
Prior parasitaemia	-0.14 (-0.4-0.11)	0.269	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.1 (0.06-0.14)	**<0.001**	247
Parasitaemia	0.44 (0.19-0.69)	**0.001**	
Alpha-thal HET	0.04 (-0.16-0.23)	0.701	
Alpha-thal HOM	-0.05 (-0.3-0.19)	0.673	
Sickle trait	-0.29 (-0.55--0.03)	**0.029**	
Prior episode	0.1 (0.01-0.19)	**0.035**	
Parasite negative[7]			
Age (years)	0.08 (0.04-0.12)	**<0.001**	213
Alpha-thal HET	0.11 (-0.09-0.31)	0.296	
Alpha-thal HOM	-0.04 (-0.29-0.2)	0.731	
Sickle trait	-0.21 (-0.47-0.05)	0.112	
Prior episode	0.12 (0.03-0.21)	**0.009**	
Parasite positive[8]			
Age (years)	0.16 (0.03-0.29)	**0.02**	34
Alpha-thal HET	-0.25 (-0.87-0.38)	0.427	
Alpha-thal HOM	0.7 (-0.53-1.93)	0.253	
Sickle trait	-1.11 (-2.24-0.03)	0.055	
Prior episode	-0.28 (-0.75-0.19)	0.234	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitaemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 3.11D Effect of different factors on IgG responses to AMA1(W2mef) in the October 2003 cross sectional bleed.

	AMA1(W2mef) Oct 2003		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.12 (0.09-0.15)	**<0.001**	294
Parasitaemia	1.14 (0.93-1.34)	**<0.001**	293
Alpha-thal HET	-0.08 (-0.27-0.12)	0.436	285
Alpha-thal HOM	-0.29 (-0.54--0.05)	**0.02**	
Sickle trait	-0.23 (-0.48-0.03)	0.078	284
Prior parasitaemia	0.24 (0.14-0.34)	**<0.001**	261
Prior episode	0.24 (0.09-0.39)	**0.002**	261
Multivariate (previous parasitaemia)[6]			
All samples			
Age (years)	0.11 (0.08-0.14)	**<0.001**	248
Parasitaemia	0.95 (0.74-1.15)	**<0.001**	
Alpha-thal HET	-0.09 (-0.25-0.07)	0.259	
Alpha-thal HOM	-0.11 (-0.31-0.1)	0.298	
Sickle trait	-0.28 (-0.48--0.07)	**0.008**	
Prior parasitaemia	0.17 (0.09-0.25)	**<0.001**	
Parasite negative[7]			
Age (years)	0.1 (0.07-0.13)	**<0.001**	212
Alpha-thal HET	-0.06 (-0.22-0.11)	0.504	
Alpha-thal HOM	-0.04 (-0.23-0.16)	0.721	
Sickle trait	-0.12 (-0.33-0.08)	0.24	
Prior parasitaemia	0.2 (0.12-0.29)	**<0.001**	
Parasite positive[8]			
Age (years)	0.21 (0.07-0.36)	**0.005**	36
Alpha-thal HET	-0.22 (-0.76-0.31)	0.402	
Alpha-thal HOM	-0.34 (-1.42-0.74)	0.522	
Sickle trait	-1.12 (-1.81--0.43)	**0.002**	
Prior parasitaemia	0.12 (-0.17-0.42)	0.402	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.11 (0.08-0.14)	**<0.001**	248
Parasitaemia	1.01 (0.8-1.21)	**<0.001**	
Alpha-thal HET	-0.09 (-0.25-0.08)	0.29	
Alpha-thal HOM	-0.08 (-0.29-0.13)	0.451	
Sickle trait	-0.27 (-0.48--0.06)	**0.011**	
Prior episode	0.19 (0.08-0.31)	**0.001**	
Parasite negative[7]			
Age (years)	0.1 (0.07-0.13)	**<0.001**	212
Alpha-thal HET	-0.07 (-0.24-0.1)	0.42	
Alpha-thal HOM	-0.03 (-0.23-0.18)	0.776	
Sickle trait	-0.1 (-0.31-0.11)	0.361	
Prior episode	0.21 (0.09-0.33)	**0.001**	
Parasite positive[8]			
Age (years)	0.21 (0.07-0.34)	**0.004**	36
Alpha-thal HET	-0.19 (-0.7-0.33)	0.464	
Alpha-thal HOM	-0.18 (-1.2-0.85)	0.725	
Sickle trait	-1.18 (-1.85--0.51)	**0.001**	
Prior episode	0.22 (-0.2-0.64)	0.288	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitaemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 3.11E Effect of different factors on IgG responses to AMA1(W2mef) in the May 2004 cross sectional bleed.

	AMA1(W2mef) May 2004		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.1 (0.07-0.13)	**<0.001**	279
Parasitaemia	0.87 (0.57-1.17)	**<0.001**	280
Alpha-thal HET	-0.03 (-0.21-0.14)	0.705	261
Alpha-thal HOM	-0.31 (-0.53--0.09)	**0.006**	
Sickle trait	-0.2 (-0.43-0.03)	0.081	266
Prior parasitaemia	0.24 (0.06-0.41)	**0.008**	254
Prior episode	-	-	
Multivariate (previous parasitaemia)[6]			
All samples			
Age (years)	0.09 (0.06-0.13)	**<0.001**	239
Parasitaemia	0.56 (0.25-0.87)	**<0.001**	
Alpha-thal HET	-0.03 (-0.19-0.14)	0.729	
Alpha-thal HOM	-0.2 (-0.41-0)	0.055	
Sickle trait	-0.2 (-0.41-0.02)	0.071	
Prior parasitaemia	0.16 (0-0.33)	**0.05**	
Parasite negative[7]			
Age (years)	0.09 (0.05-0.12)	**<0.001**	224
Alpha-thal HET	-0.02 (-0.18-0.15)	0.85	
Alpha-thal HOM	-0.18 (-0.39-0.03)	0.088	
Sickle trait	-0.2 (-0.42-0.02)	0.068	
Prior parasitaemia	0.23 (0.04-0.41)	**0.019**	
Parasite positive[8]			
Age (years)	0.23 (-0.04-0.51)	0.086	15
Alpha-thal HET	-0.52 (-1.7-0.67)	0.348	
Alpha-thal HOM	-1.42 (-3.4-0.56)	0.139	
Sickle trait	-0.29 (-2-1.41)	0.708	
Prior parasitaemia	0.1 (-0.68-0.88)	0.782	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.1 (0.07-0.13)	**<0.001**	239
Parasitaemia	0.61 (0.31-0.92)	**<0.001**	
Alpha-thal HET	-0.04 (-0.21-0.12)	0.595	
Alpha-thal HOM	-0.21 (-0.42-0)	**0.046**	
Sickle trait	-0.19 (-0.41-0.02)	0.074	
Prior episode	-	-	
Parasite negative[7]			
Age (years)	0.09 (0.06-0.12)	**<0.001**	224
Alpha-thal HET	-0.03 (-0.2-0.14)	0.748	
Alpha-thal HOM	-0.18 (-0.39-0.03)	0.088	
Sickle trait	-0.19 (-0.41-0.03)	0.083	
Prior episode	-	-	
Parasite positive[8]			
Age (years)	0.21 (0.01-0.41)	**0.04**	15
Alpha-thal HET	-0.57 (-1.62-0.48)	0.256	
Alpha-thal HOM	-1.49 (-3.27-0.28)	0.09	
Sickle trait	-0.41 (-1.73-0.9)	0.501	
Prior episode	-	-	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitaemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 3.11F Effect of different factors on IgG responses to AMA1(W2mef) in the October 2004 cross sectional bleed.

	AMA1(W2mef) Oct 2004		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.07 (0.05-0.09)	**<0.001**	273
Parasitaemia	0.55 (0.24-0.86)	**0.001**	273
Alpha-thal HET	-0.02 (-0.16-0.13)	0.838	268
Alpha-thal HOM	-0.17 (-0.36-0.01)	0.067	
Sickle trait	-0.11 (-0.29-0.07)	0.239	270
Prior parasitaemia	0.23 (-0.12-0.58)	0.196	235
Prior episode	-	-	
Multivariate (previous parasitaemia)[6]			
All samples			
Age (years)	0.09 (0.06-0.12)	**<0.001**	216
Parasitaemia	0.62 (0.25-0.99)	**0.001**	
Alpha-thal HET	-0.03 (-0.17-0.12)	0.736	
Alpha-thal HOM	-0.17 (-0.36-0.01)	0.062	
Sickle trait	-0.14 (-0.33-0.04)	0.121	
Prior parasitaemia	0.24 (-0.08-0.56)	0.137	
Parasite negative[7]			
Age (years)	0.09 (0.06-0.12)	**<0.001**	209
Alpha-thal HET	-0.05 (-0.19-0.1)	0.531	
Alpha-thal HOM	-0.19 (-0.38--0.01)	**0.042**	
Sickle trait	-0.1 (-0.29-0.08)	0.274	
Prior parasitaemia	0.25 (-0.07-0.57)	0.126	
Parasite positive[8]			
Age (years)	0.12 (-0.42-0.65)	0.45	7
Alpha-thal HET	0.28 (-3.03-3.6)	0.75	
Alpha-thal HOM	0.56 (-3.7-4.83)	0.626	
Sickle trait	-0.69 (-3.84-2.45)	0.443	
Prior parasitaemia	-	-	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.09 (0.06-0.12)	**<0.001**	216
Parasitaemia	0.62 (0.25-0.98)	**0.001**	
Alpha-thal HET	-0.02 (-0.16-0.13)	0.834	
Alpha-thal HOM	-0.16 (-0.34-0.03)	0.091	
Sickle trait	-0.14 (-0.33-0.04)	0.12	
Prior episode	-	-	
Parasite negative[7]			
Age (years)	0.09 (0.06-0.12)	**<0.001**	209
Alpha-thal HET	-0.04 (-0.18-0.11)	0.623	
Alpha-thal HOM	-0.17 (-0.36-0.01)	0.064	
Sickle trait	-0.1 (-0.29-0.08)	0.273	
Prior episode	-	-	
Parasite positive[8]			
Age (years)	0.12 (-0.42-0.65)	0.45	7
Alpha-thal HET	0.28 (-3.03-3.6)	0.75	
Alpha-thal HOM	0.56 (-3.7-4.83)	0.626	
Sickle trait	-0.69 (-3.84-2.45)	0.443	
Prior episode	-	-	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitaemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

3.3.6.2 Multivariable analysis of factors associated with IgG responses

Age, parasite status at sample time, and recorded episodes of parasitaemia and clinical malaria in the prior six months all affected antibody titres significantly throughout the study period ($p<0.001$); therefore they were all included in the multivariable linear regression. Though not as marked in their effect on antibody levels; both sickle trait and alpha-thalassemia were included in the model as well due to their protective effect against clinical malaria. Comparison of multivariable linear models showed that the best fitting model was one with all the dependent variables above included in the ways in which they had first been designated; age as a continuous variable, parasitaemia as a binary variable, and the rest as categorical variables. As prior clinical malaria episodes are a subset of parasitaemic episodes, they were fitted into the model separately.

In each multivariable model that included prior parasitaemia as a cofactor, concurrent parasitaemia had the greatest effect on IgG responses to AMA1 and MSP2 variants at every cross sectional bleed (all p <0.001). Maximal β coefficients observed due to concurrent parasitaemia were 0.95 for AMA1(W2mef) in October 2003(Table 3.11D), 0.89 for AMA1(HB3) in October 2003 (Appendix 1 Table 33D), 0.86 for AMA1(3D7) in October 2003 (Appendix 1 Table 34D), 1.25 for MSP2(3D7) in October 2004 (Appendix 1 Table 35F), and 0.70 for MSP2(FC27) in October 2002 (Appendix 1 Table 36B). Concurrent parasitaemia was followed by prior parasitaemia and age as the factors with significant positive effects on antibody levels (Tables 3.11A-3.11F, Appendix 1 Tables 33A-36F).

Due to the fact that presence of parasitaemia at the time of sampling is associated with higher antibody levels and may confound the effects of other variables, the multivariable linear regression was stratified by parasite infection status. In the case of children with concurrent parasitaemia at sample time the multivariable model could not reliably predict the effect of the dependent variables on antibody levels due to the small number of children with concurrent parasitaemia at each bleed (Tables 3.11A-3.11F, Appendix 1 Tables 33A-36F). When there were sufficient numbers for the multivariable model to work correctly, age was consistently the factor most strongly associated ($p \leq 0.05$) with antibody levels for all the antigens tested. Increasing age was associated with a maximum unit increase in antibody levels of 0.21 to AMA1(W2mef), 0.19 to AMA1(HB3), 0.24 to

AMA1(3D7), and 0.15 to MSP2(FC27) all in October 2003 (Table 3.11D, Appendix 1 Tables 33D, 34D, 36D). Sickle trait was associated with a significant reduction ($p \leq 0.01$) in antibody titres with β coefficients of -1.12 for AMA1(W2mef), -0.99 for AMA1(HB3), -0.95 for AMA1(3D7), and -1.81 for MSP2(3D7) in October 2004 and October 2003 for all AMA1 antigens (Table 3.11D, Appendix 1 Tables 33D, 34D, and 35F). Being alpha-thalassemia homozygous was associated with a significant ($p \leq 0.05$) reduction in antibody titres with β coefficients of -0.29 in May 2002 and October 2003, and -0.31 in May 2004 for AMA1(W2mef) (Tables 3.11A, 3.11C, 3.11E). This polymorphism was also associated with significant ($p \leq 0.05$) reduction in antibody titres to AMA1(HB3) and AMA1(3D7) with β coefficients of ranging from -0.2 to -0.31 in May 2002, October 2002, October 2003, and May 2004 and those to both MSP2 variants by -0.14 to -0.24 in the same months (Appendix 1 Tables 33A-36F). Recorded incidences of parasitaemia in the 6 months prior to the sample time did not appear to have any effect on antibody titres to any antigen when parasites were present at sampling.

In children who did not have parasites at sample time, the effects of the dependent variables were different from those with concurrent parasitaemia. Both prior parasitaemia and age significantly increased ($p < 0.001$) antibody titres to all the antigens tested. Prior parasitaemia consistently increased antibody titres to a greater extent than age with maximum unit increases in May 2003 of 0.28 for AMA1(W2mef), 0.3 for AMA1(HB3), 0.27 for AMA1(3D7), 0.26 for MSP2(3D7), and 0.2 for MSP2(FC27) (Table 3.11C, Appendix 1 Tables 33C, 34C, 35C, and 36C). The effect of age, though significant ($p < 0.001$), was lower than that of prior parasitaemia with maximum unit increases also occurring during the May 2003 cross-sectional bleed of 0.07 for AMA1(W2mef), 0.08 for AMA1(HB3), 0.07 for AMA1(3D7), and 0.06 for MSP2(3D7) (Table 3.11C, Appendix 1 Tables 33C, 34C, and 35C). Although both the sickle trait and being homozygous for alpha-thalassemia reduced antibody levels to both AMA1 and MSP2 in children without concurrent parasitaemia, this effect was not significant.

When prior clinical episodes were substituted for prior episodes of parasitaemia in the above mentioned models, the same effects were seen for all antigens. Age had the most significant effect in increasing antibody titres in children with concurrent parasitmeia whereas prior episodes were

most important in children without parasites at sample time (Tables 3.11A-3.11F, Appendix 3 Tables 33A-36F). These results indicate that age, prior parasitaemia/clinical malaria, and concurrent parasitaemia at sample time all have independent effects on antibody titres to AMA1 and MSP2 in this cohort.

In summary, concurrent parasitaemia had the greatest effect on raising IgG responses to AMA1 and MSP2 regardless of the levels of malaria transmission at the time of sampling (Table 3.12). Prior parasitaemia (or episodes) also positively affected IgG responses, but this effect was much more pronounced in aparasitaemic children. The positive effect of increasing age on IgG responses to AMA1 and MSP2 depended on both the infection status of the child at sampling and also the levels of malaria transmission at sampling. When transmission was high, age played a smaller role in raising IgG levels in parasitaemic children. The effect of age was similar in aparasitaemic children at high malaria transmission intensities as in both parasitaemic and aparasitaemic children at lower transmission intensities. Presence of the sickle trait or being homozygous for alpha-thalassemia reduced the IgG response to AMA1 and MSP2 in all cases although this effect was greatest in parasitaemic children at higher malaria transmission intensities (Table 3.12).

Table 3.12 Summary of the effect of various factors on IgG responses at different malaria transmission intensities in parasitaemic and aparasitaemic children.

	Malaria transmission level[Δ]			
	'High'		'Low'	
	Parasitaemic[ε]	Aparasitaemic[ε]	Parasitaemic[ε]	Aparasitaemic[ε]
Concurrent parasitaemia[1]	↑↑↑↑↑	-	↑↑↑↑↑	-
Prior parasitaemia[2]	↑	↑↑↑↑	↑↑	↑↑↑
Age[3]	↑↑	↑↑↑	↑↑↑	↑↑↑
Haemoglobinopathies[4]	↓↓↓↓	↓↓↓	↓↓↓	↓↓

Notes
[Δ] Malaria transmission levels indicated by parasite prevalence at cross sectional bleed.
[ε] Status of the child at sampling.
[1] Concurrent parasitaemia at sampling.
[2] Recorded parasitaemia in the 6 months prior to sampling.
[3] Age in years.
[4] Presence of sickle trait or being homozygous for alpha-thalassemia.
↑ Raises IgG levels (number of arrows is indicative of the strength of the effect).
↓ Lowers IgG levels (number of arrows is indicative of the strength of the effect).

3.3.7 Increased prior *P. falciparum* exposure does not always result in a high titre antibody response to AMA1 and MSP2

In order to assess how likely prior exposure to *P. falciparum* was going to result in high IgG responses, the probability of antibody levels above the 75th centile were estimated by logistic regression by fitting the number of prior parasitaemic episodes along with age as covariates. The logits of these models were then converted into probabilities and plotted to compare the effects of increasing prior exposure on antibody levels. These probabilities were worked out for all samples as well as stratified by parasite status at sampling time and by the age of the children at sampling. Details of the number of parasitaemic episodes that were recorded in the 6 months prior to sampling can be found in Table 2.3. Briefly, the proportion of children who did not have any parasitaemic episodes in the 6 months prior to sampling ranged from 48% before the May 2003 sampling to 97.5% before the Ocotber 2004 sampling. Those with 1-3 episodes ranged from 2.6% in before the October 2004 sampling to 45.7% before the May 2003 sampling. Children with 4-5 episodes ranged from 0.8% (2 children) before the October 2003 sampling to 5.5% (14 children) before the May 2003 sampling. Only 2 children had 7 parasitaemic episodes in the 6 months prior to the October 2003 cross sectional bleed.

For all antigens (three AMA1 and two MSP2 variants), the probability of antibody levels above the 75th centile increased in a linear manner from 20% when no parasitaemic episodes were experienced in the prior 6 months to 43% for AMA1(HB3), AMA1(3D7), and MSP2(FC27) after 2 recorded episodes of parasitaemia in the prior 6 months (Figure 3.8A). The probabilities of a high antibody response to AMA1(W2mef) and MSP2(3D7) also rose from 20% without any prior exposure to 40% after 2 recorded parasitaemic episodes. The probabilities of developing a high response to these five antigens changed on further exposure. The probability of a high response to AMA1(3D7) continued to rise up to 47% after 5 recorded episodes compared to the others. Regardless of further exposure, the probability of high responses to AMA1(W2mef) and AMA1(HB3) remained at 40% even after 5 episodes (Figure 3.8A). Following a peak of 45% probability of a high response to MSP2(3D7) after 3 recorded episodes which dropped to 40% on

the fourth episode, the probability of a high anti-MSP2(3D7) response dropped slowly to 37% after 7 recoded episodes (Figure 8A). The probability of a high response to MSP2(FC2) also dropped on further exposure to 37% after 4 episodes. Regardless of further exposure, the probability of high responses to AMA1(W2mef) and AMA1(HB3) remained at 40% even after 5 episodes (Figure 3.8A). This may indicate that there is s subgroup of children who are non-responsers.

A similar pattern was seen for the children who were parasite negative at the time of sample collection. Probabilities rose in association with increasing numbers of episodes at a similar rate for all antigens and then began to separate after 2 recorded episodes from 30-35% (Figure 3.8C). The probability of a high anti-AMA1(3D7) response continued to rise on further exposure to 45% after 7 recorded episodes of parasitaemia. In children who were parasitaemic at sample time, the probability of antibody titres above the 75th centile when there were no prior episodes recorded was between 60% and 70% for all antigens tested. These probabilities declined at a similar constant rate for all three AMA1 variants to 43-48% after 7 recorded episodes of parasitaemia (Figure 3.8B). The probability of a high response to MSP2(FC7) declined at a slower rate than that of other antigens from a high of 70% when there were no prior episodes to 60% after 2 episodes and remained relatively stable with a slight decline to 58% after 7 episodes. The probability of a high response to MSP2(3D7) was different to that of MSP2(FC27). The probability declined rapidly from 67% at baseline to 38% after 4 episodes and remained constant even on further exposure (Figure 3.8B). These observations suggest that if children were parasitaemic at sample collection time, antibodies were at high levels and were not further increased by a history of prior episodes.

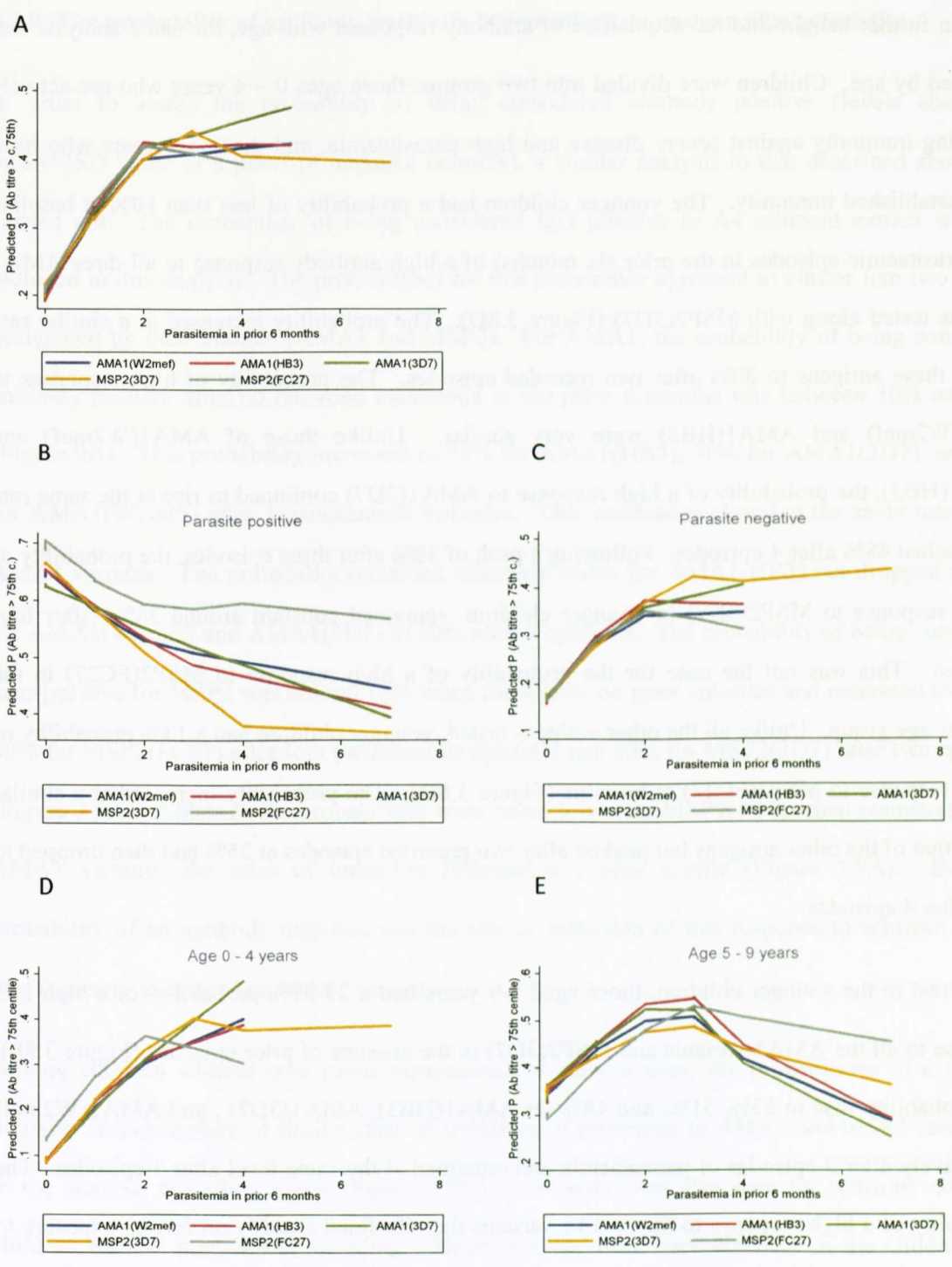

Figure 3.8 The predicted probability of IgG responses to *P. falciparum* merozoite antigens above the 75th centile by the number of recorded parasitaemic episodes in the 6 months prior to sampling. Each antigen is represented by a single line on each graph; dark blue = AMA1(W2mef), maroon = AMA1(HB3), green = AMA1(3D7), yellow = MSP2(3D7), light blue = MSP2(FC27). (A) Predicted probability of antibody titres above the 75th centile in all samples, (B) parasite positive at sample time, and (C) parasite positive at sample time. Predicted probability of antibody titres above the 75th centile in children ages 0-4 years (D) and ages 5-9 years (E) at sampling.

To gain further insight into the acquisition of antibody responses with age, the same analysis was stratified by age. Children were divided into two groups; those ages 0 – 4 years who are actively acquiring immunity against severe disease and high parasitaemia, and those 5-9 years who have more established immunity. The younger children had a probability of less than 10% at baseline (no parasitaemic episodes in the prior six months) of a high antibody response to all three AMA1 variants tested along with MSP2(3D7) (Figure 3.8D). The probability increased at a similar rate for all these antigens to 30% after two recorded episodes. The probability of high responses to AMA(W2mef) and AMA1(HB3) were very similar. Unlike those of AMA1(W2mef) and AMA1(HB3), the probability of a high response to AMA1(3D7) continued to rise at the same rate and reached 48% after 4 episodes. Following a peak of 38% after three episodes, the probability of a high response to MSP2(3D7) in younger children remained constant around 35% after four episodes. This was not the case for the probability of a high response to MSP2(FC27) in the younger age group. Unlike all the other antigens tested, younger children had a 13% probability of a high response to MSP2(FC27) at baseline (Figure 3.8D). The probability increased at a similar rate to that of the other antigens but peaked after two recorded episodes at 35% and then dropped to 28% after 4 episodes.

In contrast to the younger children, those aged 5-9 years had a 33-37% probability of a high IgG response to all the AMA1 variants and MSP2(3D7) in the absence of prior episodes (Figure 3.8E). The probability rose to 53%, 51%, and 48% for AMA1(HB3), AMA1(3D7) , and AMA1(W2mef) respectively after 3 episodes of parasitaemia and remained at the same level after 3 episodes. The probability of a high response to the AMA1 variants then declined steadily on further exposure to 28% for AMA1(W2mef) and AMA1(HFB3), and 25% for AMA1(3D7) after seven episodes. The probability of a high response to MSP2(3D7) had a similar profile to that of the AMA1 variants but with more modest increases and declines (Figure 3.8E). Following a peak of 46% after four episodes, the probility of a high response to MSP2(3D7) declined to levels similar to that at baseline (34%) after seven episodes.

3.3.8 The probability of antibody positivity is dependent on antigen characteristics

In order to assess the probability of being considered antibody positive (levels above the mean+3SD value of a panel of negative controls), a similar analysis to that described above was carried out. The probability of being considered IgG positive to A4 schizont extract was also included in this analysis. The probabilities for this occurrence appeared to cluster into two groups designated by their antigen (AMA1 and MSP2). For AMA1, the probability of being considered antibody positive after no recorded parsitemia in the prior 6 months was between 30% and 45% (Figure 9A). This probability increased to 75% for AMA1(HB3), 70% for AMA1(3D7), and 65% for AMA1(W2mef) after 3 parasitaemic episodes. This increase occurred at the same rate for all AMA1 variants. The probability remained relatively stable for AMA1(HB3) but dropped slightly for AMA1(W2mef) and AMA1(3D7) to 50% after 7 episodes. The probability of being considered sero-positive for MSP2 was around 15% when there were no prior episodes and increased to almost 40% for MSP2(FC27) after four parasitaemic episodes and 30% for MSP2(3D7) after two episodes (Figure 3.9A). Although the probabilities were much lower for MSP2(3D7) when compared to the AMA1 variants, the rates of induction followed a similar profile (Figure 3.9A). Both the probability of an antibody response and the rate of induction of this response to schizont extract was the same as that to the AMA1 variants.

Among children without concurrent parasitaemia at sample time, the probabilities of a positive antibody response showed similar rates of induction of responses to AMA1 and MSP2 variants as in the analysis described above (Figure 3.9C). The same was also seen for schizont extract in children without parasites at sampling. These patterns were very different in the children with concurrent parasitaemia (Figure 3.9B). The probability of a positive antibody response to all three AMA1 variant was 78-83% when there were no prior episodes. Regardless of the number of episodes of prior exposure, the probability of being antibody positive to AMA1(W2mef) did not change. This was in contrast with AMA1(HB3) and AMA1(3D7). The probabilities of an antibody response to these two antigens declined steadily on every exposure to 60% for AMA1(3D7) and 50% for AMA1(HB3) after seven prior episodes of parasitaemia (Figure 3.9B). The probability of an antibody response to MSP2 was between 57% and 60% without prior

episodes, which was lower than that for AMA1. Whereas this probability did not change markedly for MSP2(FC27) even after 7 episodes, the probability of an antibody response to MSP2(3D7) declined to 40% by four episodes and remained steady even after more episodes of parasitaemia. The probability of an antibody response to schizont extract was around 80% at baseline and increased to 90% after 2 episodes (Figure 3.9B). It then declined steadily to baseline levels after seven episodes.

Stratification by age, as described in the earlier section gave further insight into the induction of antibody responses to AMA1 and MSP2. Children ages 0 – 4 years had a similar pattern of induction to that seen when all the samples were analysed together (Figure 3.9D). Younger children had probabilities between 18% and 23% of an antibody response to the AMA1 variants and schizont extract when no prior episodes were recorded. The rates of induction increased at a similar rate and peaked after 3 episodes for AMA1(W2mef), AMA1(3D7), and schizont extract at 60%, 60%, and 70% respectively. The rate of induction of an antibody response to AMA1(HB3) slowed after 3 episodes but continues to rise to 80% after 7 episodes (Figure 3.9D). The probability of an antibody response to AMA1(W2mef) remained steady after 3 episodes but declined to 50% for AMA1(3D7). More than three episodes resulted in a similar decline in the probability of an antibody response to schizont extract. Though the probability of antibody responses to the MSP2 variants were lower than AMA1 when there were no prior episodes (less than 10%), they rose at the same rate to around 20% after two episodes of parasitaemia and then remained steady on further exposure (Figure 3.9D).

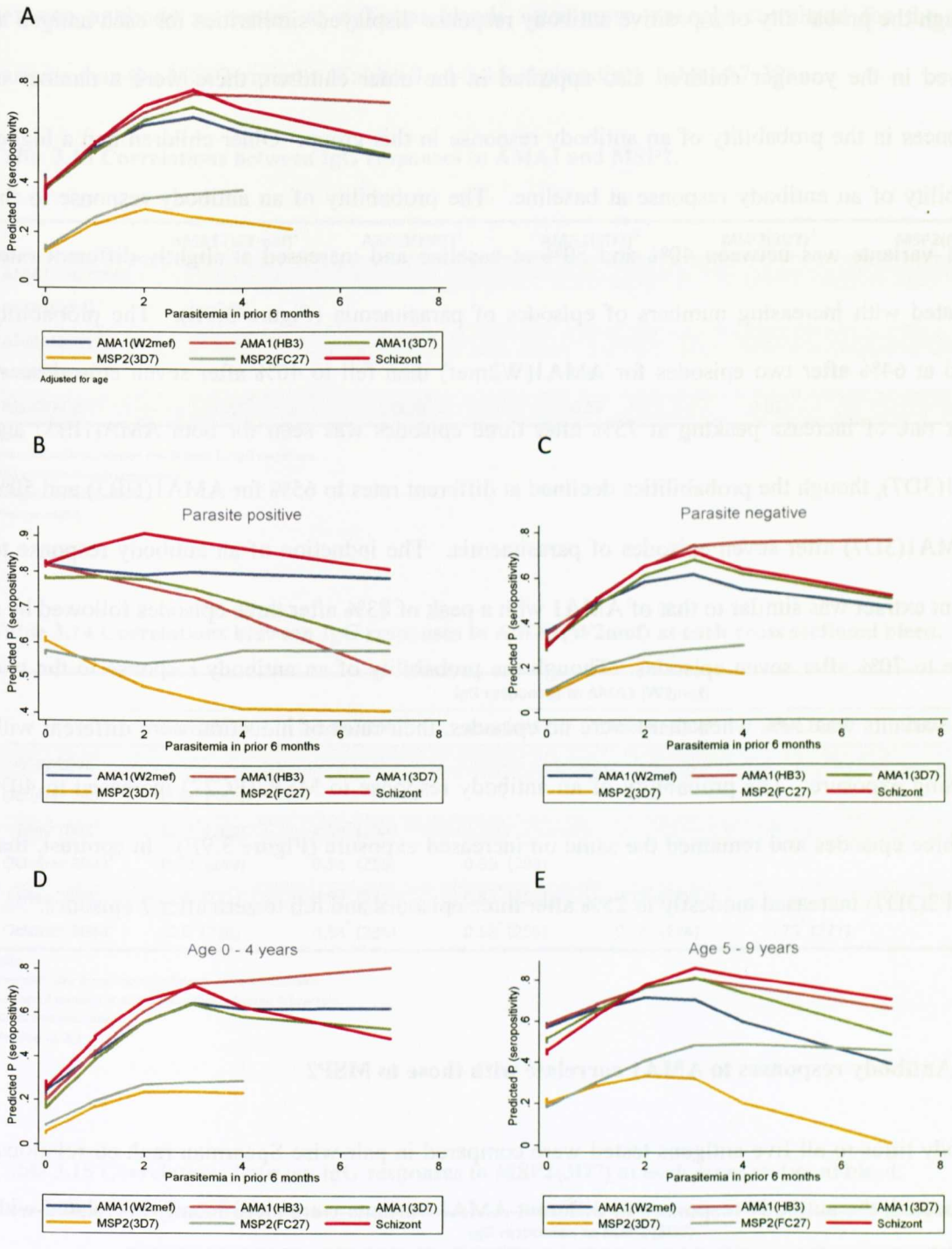

Figure 3.9 The predicted probability of positive IgG responses to *P. falciparum* merozoite antigens by the number of recorded parasitaemic episodes in the 6 months prior to sampling. Each antigen is represented by a single line on each graph; dark blue = AMA1(W2mef), maroon = AMA1(HB3), green = AMA1(3D7), yellow = MSP2(3D7), light blue = MSP2(FC27), red = A4 schizont extract. (A) Predicted probability of sero-positive responses in all samples, (B) parasite positive at sample time, and (C) parasite positive at sample time. Predicted probability of sero-positive responses in children ages 0-4 years (D) and ages 5-9 years (E) at sampling.

Although the probability of a positive antibody response displayed similarities for each antigen as observed in the younger children also appeared in the older children, there were a number of differences in the probability of an antibody response in this group. Older children had a higher probability of an antibody response at baseline. The probability of an antibody response to the AMA1 variants was between 40% and 50% at baseline and increased at slightly different rates associated with increasing numbers of episodes of parasitaemia (Figure 3.9E). The probability peaked at 64% after two episodes for AMA1(W2mef) then fell to 40% after seven episodes. A similar rate of increase peaking at 75% after three episodes was seen for both AMA(HB3) and AMA1(3D7), though the probabilities declined at different rates to 65% for AMA1(HB3) and 50% for AMA1(3D7) after seven episodes of parasitaemia. The induction of an antibody response to schizont extract was similar to that of AMA1 with a peak of 83% after three episodes followed by a decline to 70% after seven episodes. Though the probability of an antibody response to the two MSP2 variants was 20% when there were no episodes, their rates of induction were different with increasing exposure. The probability of an antibody response to MSP2(FC27) increased to 40% after three episodes and remained the same on increased exposure (Figure 3.9E). In contrast, that to MSP2(3D7) increased modestly to 25% after three episodes and fell to zero after 7 episodes.

3.3.9 Antibody responses to AMA1 correlate with those to MSP2

Antibody titres to all five antigens tested were compared in pair-wise Spearman rank correlations. As expected, the antibody responses to different AMA1 variants were significantly correlated with correlation coefficients between 0.87 and 0.91. The response to the two MSP2 variants was not as strongly correlated, with a correlation coefficient of 0.62 though this was highly significant ($p<0.001$) (Table 3.13). This may indicate that antibodies to MSP2 are more allele-specific than antibodies to AMA1 (Stanisic et al. 2009). High antibody responses to AMA1 were all associated with a comparable level of anti-MSP2 responses with significant ($p<0.001$) correlation coefficients from 0.59 to 0.64 for AMA1/MSP2 pair-wise comparisons. Spearman rank correlations comparing the IgG response to the same antigen at each cross sectional bleed showed that the antibody responses at consecutive bleeds were more strongly associated with each other. Correlations

between antibody responses at different bleeds were more strongly correlated for the AMA1 variants than the MSP2 variants (Tables 3.14-3.15, Appendix 1 Tables 37-39).

Table 3.13 Correlations between IgG responses to AMA1 and MSP2.

	AMA1 (W2mef)[1]	AMA1(HB3)[1]	AMA1(3D7)[1]	MSP2(3D7)[1]	MSP2(FC27)[1]
AMA1 (W2mef)[1]	-	-	-	-	-
AMA1(HB3)[1]	0.89*	-	-	-	-
AMA1(3D7)[1]	0.87*	0.91*	-	-	-
MSP2(3D7)[1]	0.61*	0.62	0.64*	-	-
MSP2(FC27)[1]	0.59*	0.59*	0.59*	0.62*	-

Notes
Spearman rank correlation coefficients for IgG responses.
2100 samples in each comparison.
[1] Antigens tested by ELISA.
* *P*-value <0.001

Table 3.14 Correlations between IgG responses to AMA1(W2mef) at each cross sectional bleed.

	IgG responses to AMA1 (W2mef)					
	May 2002[1]	**October 2002[1]**	**May 2003[1]**	**October 2003[1]**	**May 2004[1]**	**October 2004[1]**
May 2002[1]	-	-	-	-	-	-
October 2002[1]	0.68* (308)	-	-	-	-	-
May 2003[1]	0.57* (263)	0.53* (294)	-	-	-	-
October 2003[1]	0.59* (249)	0.51* (259)	0.59* (293)	-	-	-
May 2004[1]	0.6* (231)	0.55* (242)	0.53* (259)	0.72* (278)	-	-
October 2004[1]	0.6* (226)	0.53* (236)	0.58* (255)	0.74* (274)	0.75* (277)	-

Notes
Spearman rank correlation coefficients for IgG responses.
Number of samples in each comparison indicated in brackets.
[1] Cross sectional bleed.
* *P*-value <0.001

Table 3.15 Correlations between IgG responses to MSP2(3D7) at each cross sectional bleed.

	IgG responses to MSP2 (3D7)					
	May 2002[1]	**October 2002[1]**	**May 2003[1]**	**October 2003[1]**	**May 2004[1]**	**October 2004[1]**
May 2002[1]	-	-	-	-	-	-
October 2002[1]	0.53* (308)	-	-	-	-	-
May 2003[1]	0.47* (263)	0.59* (294)	-	-	-	-
October 2003[1]	0.42* (249)	0.49* (259)	0.53* (293)	-	-	-
May 2004[1]	0.47* (231)	0.54* (242)	0.56* (259)	0.61* (278)	-	-
October 2004[1]	0.41* (226)	0.62* (236)	0.56* (255)	0.58* (274)	0.72* (277)	-

Notes
Spearman rank correlation coefficients for IgG responses.
Number of samples in each comparison indicated in brackets.
[1] Cross sectional bleed.
* *P*-value <0.001

3.4 Discussion

Cross sectional studies are the simplest way to collect data from a population sample and make inferences on the entire population. Whilst this method is valid for observations that either remain relatively constant or change slowly over time, it may not be reliable when the outcome under question is dynamic in nature. A good example of a dynamic outcome is the antibody response to malarial antigens. Antibody responses are dependent on a number of factors that include the age of the individual as well as the level of exposure to malaria. With the extra knowledge that antibodies to various malarial antigens may be short-lived, it is important to note that methods which take into the account the dynamic nature of the outcome will likely be the best in investigating antibody responses to malarial antigens (Kinyanjui et al. 2007). The ideal framework for this outcome is one that is longitudinal (which will be discussed in later chapters), but barring the possibility of a longitudinal framework, a number of repeated cross sectional surveys will give insight into the nature of the antibody response. This study is unique in the fact that it was made up of a set of six repeated cross sectional surveys in which the same parameters were recorded in almost 300 children per survey, with close to two-thirds of them having been sampled at all six surveys. Here, I was able to identify the major factors that influence induction and maintenance of antibody responses to AMA1 and MSP2. These factors were age, recent or current malarial exposure and the presence of haemoglobinopathies.

In agreement with previously published findings antibody prevalence and titres to both AMA1 (W2mef, HB3 and 3D7) and MSP2 (3D7 and FC27) consistently rose significantly with age in this study (Taylor et al. 1998; Johnson et al. 2004; Polley et al. 2004; Cortes et al. 2005; Polley et al. 2006). This can be attributed to increasing cumulative exposure to malaria the longer individuals live in malaria endemic areas resulting in higher and possibly more durable antibody responses. Also, when responses to schizont extract (thought to reflect total *P. falciparum* blood-stage exposure) were considered, the antibody response to merozoite antigens rose with increasing exposure. Both of these results are reassuring because they confirm that the responses we were measuring were specific to *P. falciparum*. Consistent increases in antibody titres to all merozoite antigens tested with concurrent parasitaemia at sample time were observed and have also been

noted in numerous publications (Al-Yaman et al. 1995a; Bull et al. 2002; Metzger et al. 2003; Kinyanjui et al. 2004; Polley et al. 2006). The presence of parasites activates both a naïve and previously exposed immune system to produce significant levels of *P. falciparum* antigen-specific antibodies in what is probably the most measurable immune response to malaria infection.

Due to the nature of this study, I was able to investigate the effect of changing malaria transmission on the antibody responses in the same population. Whereas it is already established that antibody levels are dependent on malaria transmission; it has not been studied in the detail that was carried out in this study (Vande Waa et al. 1984; Fruh et al. 1991; Ramasamy et al. 1994; Jakobsen et al. 1997; Cavanagh et al. 1998; Giha et al. 1998; Soares et al. 1999b; John et al. 2002; Nebie et al. 2008). Here I showed that in children between the ages of 1 to 10, antibody prevalence to AMA1 and MSP2 fell in concert with reducing malaria transmission over 2.5 years when measured at 6 month intervals.

Age and previous exposure to malaria are two factors that are often difficult to separate and are usually combined when trying to explain their effect on protection from clinical disease. In many studies carried out in malaria-endemic areas, the age of a child is often used as a proxy for cumulative exposure. This may not be a correct assumption. Age alone has been shown to have an effect on incidence of malaria separate from any other malaria-related variables and also in relation to antibody responses to pre-erythrocytic and erythrocytic *P. falciparum* antigens (Baird et al. 1991; Baird et al. 1993; Kurtis et al. 2001; Hudson Keenihan et al. 2003; Keenihan et al. 2003). Contrary to the general assumption of cumulative exposure with increasing age, it may be better to assume that although exposure will increase with time spent in a malaria-endemic area, the exposure will not be homogenous in its distribution or frequency. Studies carried out in malaria-endemic areas show that the incidence of clinical disease is heterogenous and often occurs in clusters within these exposed populations (Carter et al. 2000; Creasey et al. 2004; Clark et al. 2008; Mwangi et al. 2008). Due to active surveillance of the cohort in this study, most incidences of exposure to *P. falciparum* could be caught and recorded on a week by week basis over the entire study period. With this information it was possible to investigate the effect of episodes of prior recorded exposure/parasitaemia in the 6 months before each sample time. As antibodies to malaria

antigens are generally short-lived, limiting the period of prior exposure to 6 months would hopefully include the antibody response to a recent exposure as well as any relatively long-lived responses that could be elicited in these children. Also, the decline in malaria transmission over the study period would allow for further separation of the effects of age and exposure on antibody responses to AMA1 and MSP2. It should be noted that not all episodes of asymptomatic parasitaemia may have been detected during follow-up as blood smears were only performed if the child was unwell, or if there was another clinical indication in which a blood sample was collected. Furthermore, sub-microscopic parasitaemia which is detectable by PCR-based methods does occur. Unfortunately PCR detection of parasitaemia is not available for this cohort at present.

Throughout the study period, concurrent parasitaemia at sample time was the greatest contributor to higher antibody levels. As this effect overshadowed the contribution of other factors to antibody levels, the analyses were stratified by parasite status at sample time. When this was done, the effect of age could be seen throughout the study period with increasing age being associated with higher antibody titres to AMA1 and MSP2. In children who did not have detectable parasitaemia at sample time, recorded episodes of parasitaemia and mild malaria increased antibody levels more than age at the beginning of the study period when malaria transmission was higher (parasite prevalence at sample time above 10%). As malaria transmission dropped over the study period, the effect of age on antibody levels became more pronounced. In children with concurrent parasitaemia at sample time, age had a much less obvious effect on antibody levels. It appears that the circulating parasites activate the immune response to such a degree that any effects of age are overwhelmed. As malaria transmission dropped over the study period, age began to have more of an effect even in children who were parasite positive. Age and prior exposure affect antibody titres in an independent manner that is dynamic and dependent on the overall malaria transmission intensity. When circulating parasites are present in previously exposed individuals, it could be that the antibody titres are mainly a response to concurrent parasitaemia that masks the effect of other factors. As suggested in other studies, this response in the presence of activating parasites could be an indication of the individual's overall responsiveness to malaria antigens (Bull et al. 2002; Polley et al. 2004).

Both the sickle trait and alpha-thalassemia were associated with a reduction in antibody titres though these associations were not significant at every sampling point. Comparison in different age groups also showed a trend for lower antibody titres associated with the haemoglobinopathies. The results seen in the HbAA and HbAS children are both in agreement with and contrary to other studies. A study in Kenya showed that protection from clinical malaria increased with age at a more rapid pace in HbAS children than in HbAA children, thus implying a role for HbAS is regulation of the adaptive immune response (Williams et al. 2005a). Antibody-related protection against variant surface antigens in relation to haemoglobinopathies was observed in two studies but was not in other studies (Marsh et al. 1989; Luzzi et al. 1991; Allen et al. 1992; Allen et al. 1993; Le Hesran et al. 1999). A more recent study that measured antibody titres to MSP2 (3D7 and FC27) found that Senegalese children with the sickle trait (HbAS) had significantly lower levels of total IgG, IgG1 and IgG3 than their normal counterparts (Sarr et al. 2006). Another recent study showed slightly higher levels of antibodies to AMA1(3D7) and MSP2(D2d) in adults from a low transmission setting in Burkina Faso but no difference in antibody titres to the same antigens in children living in a high transmission setting (Verra et al. 2007). The results from the Sarr *et al* study are in agreement with this one. The same antigens from the same source were used in both studies on children of similar ages (2-10 years in the Sarr *et al* compared to 0-10 years in this study) from areas of comparable transmission intensity (EIR of 9-12 in the Sarr *et al* study compared to 10 in this study). This study adds to evidence that the antibody responses to merozoite antigens in individuals with haemoglobinopathies may be lower compared to their normal counterparts. It has been suggested that lower antibody responses seen in HbAS and alpha-thalassemic individuals may be an effect of protection from clinical malaria (Bayoumi 1997). Reduced parasite densities due to erythrocyte physiology along with increased splenic clearance of defective erythrocytes could lead to a reduction in the amount of malaria antigens that are presented to B cells and lead to a lower antibody response.

Antibody responses to different AMA1 variants and MSP2 variants were significantly correlated both within the different antigenic alleles and between the different antigens. This result is expected as both AMA1 and MSP2 have conserved regions that will elicit a similar antibody response. The correlation between MSP2 and AMA1 can also be explained by exposure to a

number of *P. falciparum* antigens which results in an antibody response to all possible antigens. In order to further compare the responses to AMA1 and MSP2, the probability of a sero-positive response after recorded exposure to *P. falciparum* was investigated. Without any recorded exposure in the prior 6 months – which may not include all asymptomatic exposure – the probability of an antibody response considered positive was lower for MSP2 than for AMA1. Even after four recorded episodes of exposure the probability of a positive antibody response to MSP2 is on average 25% compared to 70% for AMA1. These results may indicate that AMA1 is more immunogenic than MSP2 in this cohort and therefore elicits a stronger response than that to MSP2; samples were tested at a lower concentration for AMA1 compared to MSP2. All antigens were expressed and purified using similar methods, were of high purity, and were coated on ELISA plates at saturating concentrations (Beeson, unpublished data).

The probability of a high antibody response (above the 75th centile) after recorded exposure was also investigated and the patterns were very similar for all AMA1 and MSP2 variants. The probability of a high antibody response increased in a linear manner in relation to number of episodes to more than 40% after three episodes of recorded exposure. When only those without parasites at sample time were considered a similar pattern was observed. When those with concurrent parasitaemia were considered alone, the probability of a high response was above 60% in the absence of prior episodes and dropped for all antigens with increasing prior exposure. These results suggest that in children with ongoing exposure, a high antibody response to AMA1 and MSP2 is likely to be generated after 3 episodes of exposure which may parallel the immunity to severe non-cerebral malaria that is generated after 2-3 episodes of disease (Gupta et al. 1999). If a child has not generated a high antibody response to AMA1 or MSP2 after three episodes, it is unlikely that they will after more episodes. The effect of concurrent parasitaemia was very clear with high probabilities of high antibody titres even without any exposure in the six months prior to sample time. The fact that more episodes of exposure were predictive of lower antibody levels may indicate that those children who were parasitaemic at sample time could include a group of more susceptible children who cannot generate a high antibody response to AMA1 and MSP2 even after considerable exposure.

Overall antigen immunogenicity appears to influence whether an individual will have a seropositive response after a number of *P. falciparum* exposures regardless of age. Both older (5 – 9 years) and younger (0 – 4 years) children were between 60% and 80% likely to have measurable antibody responses to AMA1 after three recorded parasitaemic episodes compared with 20% to 40% for MSP2. This was regardless of the fact that the likelihood of a measureable antibody response was much lower in younger children without any exposure in the prior six months compared to older children. Even though antigen characteristics will influence whether there is any antibody response, the individual's age appears to further implicate whether this response will be in the higher percentiles or not. If an older child has not produced a high level antibody response after 2-3 parasitaemeic episodes, they are less likely to produce this response on further exposure compared to their younger counterparts. These results could be indicative of the fact that older children who experience numerous parasitaemic episodes do so due to overall lower antibody and/or immune responses and may actually be the susceptible members of the population in which malaria transmission and disease actively occurs. An alternative explanation may be the shift from allele-specific to cross-reactive malaria immunity that occurs with aging. In this study both cross-reactive and allele specific antibodies were measured by ELISA. Younger children had different rates of acquiring a high antibody responses on increasing prior exposure, with similar rates for AMA1(W2mef) and AMA1(HB3) when compared to those of AMA1(3D7). There was also a difference between MSP2(3D7) and MSP2(FC27). In older children the differences tended to be antigen-specific rather than variants specific; likely explained by the existence of cross-reactive IgG responses specific to AMA1 and MSP2 respectively.

Though detailed in its approach, a limitation of this study is the small number of antigens tested. But even with this limited antigen repertoire, I have shown that the effects of different factors on antibody acquisition are dynamic in nature. We intend to extend this study with other merozoite antigens as immunity to malaria is dependent on responses to a number of antigens (Meraldi et al. 2004; Gray et al. 2007; Osier et al. 2008). As antibody levels are often used as a proxy for the immune response to malaria in protection studies, it is important to note that the extent to which age, prior exposure, concurrent parasitaemia, and haemoglobinopathies influence antibody levels to AMA1 and MSP2 also change over time. The acquisition of antibodies to malaria antigens in

childhood is also dependent on changing states of these factors. Here I show that when antibody responses are measured during higher malaria transmission intensities, the effect of age can only be clearly seen when children are aparasitaemic. Only when malaria transmission drops does the antibody response begin to better represent cumulative exposure in both parasitaemic and aparasitaemic children. Age also begins to play a bigger role in predicting antibody levels at lower transmission intensities. The fact that antibody responses are intimately linked to malaria transmission intensity means that interpretation of antibody data must include this confounder. These antibody properties are already being used in the sero-epidemiological prediction of malaria transmission intensity (Drakeley and Cook 2009). Longitudinal analysis of this data will allow for deeper understanding of the acquisition of antibody responses in this population (see Chapter 4).

4 Longitudinal patterns of IgG responses to AMA1 and MSP2

4.1 Introduction

The successful treatment of clinical malaria in children by the passive transfer of immunoglobulins from adults in the 1960s may have over-simplified the interpretation of the role of naturally acquired antibodies to malaria antigens (Cohen et al. 1961). To date, most – if not all – studies that investigate the role of antibodies in malaria immunity are founded on the hypotheses that the presence or absence of anti-malaria antibodies is reflective of immunity or lack of, and that immunity is relatively stable over the period of follow-up in question. It is most interesting that these premises persist in the face of growing evidence that antibody responses to malaria antigens often appear to be short-lived in children (i.e. present at detectable levels for only a few weeks) and that adults who move away from malaria endemic areas for extended periods are more susceptible to disease on their return to these areas (Deloron and Chougnet 1992; Branch et al. 1998; Cavanagh et al. 1998; Soares et al. 1999; Kinyanjui et al. 2007). The traditional method of measuring antibody responses at baseline and then associating them with protection or susceptibility over follow-up is both logistically and statistically simpler than multiple sampling over an extended period, which may contribute to the popularity of these studies. The varying results seen in comparable studies using the same antigens along with disappointing results from vaccine trials may indicate some unreliability for these types of studies to assign immune status. A more complete picture of the development of malaria immunity will be by repeated sampling and surveillance of the same individuals over an extended period of time.

Longitudinal studies allow the individual development of dynamic outcome variables to be studied over time. A number of longitudinal studies investigating the acquisition of various antibodies to malarial antigens in endemic populations have been carried out in children (Fruh et al. 1991; Branch et al. 1998; Cavanagh et al. 1998; Giha et al. 1999; Udhayakumar et al. 2001; Kinyanjui et

al. 2007; Akpogheneta et al. 2008; Biswas et al. 2008). To date two studies – both in adults have shown a stable persistence of antibody levels to MSP1 and AMA1 (Riley et al. 1993; Udhayakumar et al. 2001). Most of the studies showed that antibodies to *Pf*MSP1, *Pv*MSP1, *Pf*AMA1, *Pv*AMA1 and *Pf*EMP1 are frequently short-lived and decline significantly over the study period although antibodies to *Pv*AMA1 could be detected in a few individuals up to 7 years after an outbreak of *P. vivax* malaria in Brazil (Branch et al. 1998; Cavanagh et al. 1998; Giha et al. 1999; Soares et al. 1999; Morais et al. 2006; Kinyanjui et al. 2007; Akpogheneta et al. 2008; Biswas et al. 2008). Whilst the studies above have shown that antibody responses to merozoite antigens are often short-lived and boosted by concurrent parasitaemia or recent infection, they have not examined the acquisition of antibody responses in children below the age of 10 years over an extended period. Also lacking in published literature is the effect of changing covariates such as age and exposure on the longitudinal patterns of antibody acquisition and maintenance.

As shown in the previous chapter, antibody responses to AMA1 and MSP2 are dynamic and are affected by a number of different factors. Age, concurrent parasitaemia, and prior exposure all had considerable effects on the antibody levels exhibited by children in this study. Utilisation of both bi-annual antibody measures and weekly surveillance data over 2.5 years allows for patterns of antibody acquisition and maintenance to be examined in depth. The effects of increasing age, variable incidents of concurrent parasitaemia, and overall exposure over a period with declining malaria transmission can now be investigated more fully. Antibody levels at a single time point are not only a result of the factors already mentioned, but are also affected by antibody levels previously exhibited by the same individual (unless measured after primary *Plasmodium* exposure, which is difficult to assess in endemic populations). Therefore, what may be described as a high response at a single time point may not be the case over a longer period of time. The opposite could also be true; perhaps antibody measures at a single time point could give great insight into their magnitude over time.

This chapter aims to investigate the acquisition and maintenance of antibody responses to AMA1 and MSP2 as well as the factors affecting them throughout the study period. As already stated, both these antigens elicit antibody responses in malaria-endemic populations and are likely to be targets of protective immunity. It is hoped that the results from this chapter may shed more light

on the dynamic antibody response that is typical of naturally acquired malaria immunity and will aid in the interpretation of the sero-epidemiological data that is used as a measure of immunity in ongoing observational studies and malaria vaccine trials.

4.2 Methods

4.2.1 Statistical Analysis

All data analyses were performed with STATA version 9.2 (StataCorp, College Station, TX USA). All samples were used in the analyses unless stated. Tracking is used to investigate the stability of outcome variables over time. Rather than calculating a tracking coefficient for each individual, a simpler method of visualizing relative change over time was used. I analysed an individual's antibody response as a rank within the study population and investigated its stability over the study period using adjusted *z*-scores (the standard normal deviate). The equation used is described in Chapter 2. The *z*-score was adjusted for age and sample time to take into account the increasing age of the children over the study period and the reduction in malaria transmission over the same. Stability of the IgG response over the study period was assessed by calculating the difference in *z*-scores for each child from bleed to bleed.

In order to reduce the large number of data points at each time point to a longitudinal pattern, mean antibody levels were calculated and graphed using locally weighted scatter-plot smoothing (LOWESS). LOWESS fits a low-degree polynomial to a determined subset of the data at each point with explanatory variable values near the point where the response is being estimated (Cleveland 1979; Cleveland and Devlin 1988). 80% of the data at each point was used to fit each local polynomial using weighted least squares which gives less weight to points further away from the point whose response is being estimated. LOWESS does not require the specification of a function to fit a model to all of the data in the sample and requires large densely sampled data sets to produce good models. With over 250 data points at each sample point, this model gave a reasonable population mean.

To test for differences in antibody levels over the study period MANOVA for repeated measures was carried out. This statistical test is carried out for the $T - 1$ absolute differences between subsequent measurements. Details of this test can be found in Chapter 2. MANOVA for repeated measures assumes that observations on different subjects at each of the time points are independent and are multivariate normally distributed.

To investigate the effect of different predictor variables on antibody levels, generalised estimating equations (GEE) were used. GEE allow for the analysis of longitudinal relationships using all available longitudinal data without summarizing the longitudinal outcome into a single value. This model allows for simultaneous analysis between variables at different time points. Repeated observations on the same subject are not independent so a correction needs to be made for this within-subject correlation. GEE was also used to investigate the decline in antibody responses between May and October 2004. Details can be found in Chapter 2.

Antibody measures were entered into the model as continuous variables, age was a categorical variable defined by age at the start of the study, parasitaemia was also a categorical variable defined by the number of times an individual had concurrent parasitaemia at sample time. Prior exposure was a count variable defined by the number of times each individual had an episode of recorded parasitaemia in six months prior to sample time. Sickle trait and alpha-thalassemia were both entered as categorical variables.

4.3 Results

4.3.1 Fluctuating IgG responses to AMA1 and MSP2 parallel the decline in malaria transmission

In order to get a clearer picture of antibody patterns over the study period; standardized measures of relative antibody levels were plotted over the entire period for each child present in the study (Figure 4.1). For ease of interpretation, the sample time (May 2002 to October 2004) was indicated on the x-axis rather than the actual date of sample collection (which varied widely for each cross-sectional sample time due to the number of study participants involved). These individual antibody plots revealed a number of occurrences. The overall antibody patterns for AMA1 were different from those of MSP2 within the same group of children. A small number of children maintained a high antibody response to all variants of AMA1 throughout the study period (Figure 4.1A-C) compared to MSP2 (Figure 4.1D-E). A second group of children had highly fluctuating antibody responses that spanned the upper and lower limits of detection for both AMA1 and MSP2 variants, though these patterns were exhibited less with regards to MSP2 compared to AMA1. For both AMA1 and MSP2 there were a considerable number of children who maintained a low antibody response (below 0.5 OD units) over the entire study period compared to other children. Though there was some fluctuation in the antibody response in these children, it was small. Overall, the antibody response to AMA1 appeared to be stronger than that to MSP2, with more children exhibiting OD values to AMA1 greater than 1 OD unit compared to MSP2 (anti-AMA1 responses were tested at a sera dilution of 1:4000 and those to MSP2 at a 1:1000 dilution). When the same data was plotted for those 186 children who were present at all six cross-sectional bleeds the same patterns described above were evident (Appendix 2, Figure 1).

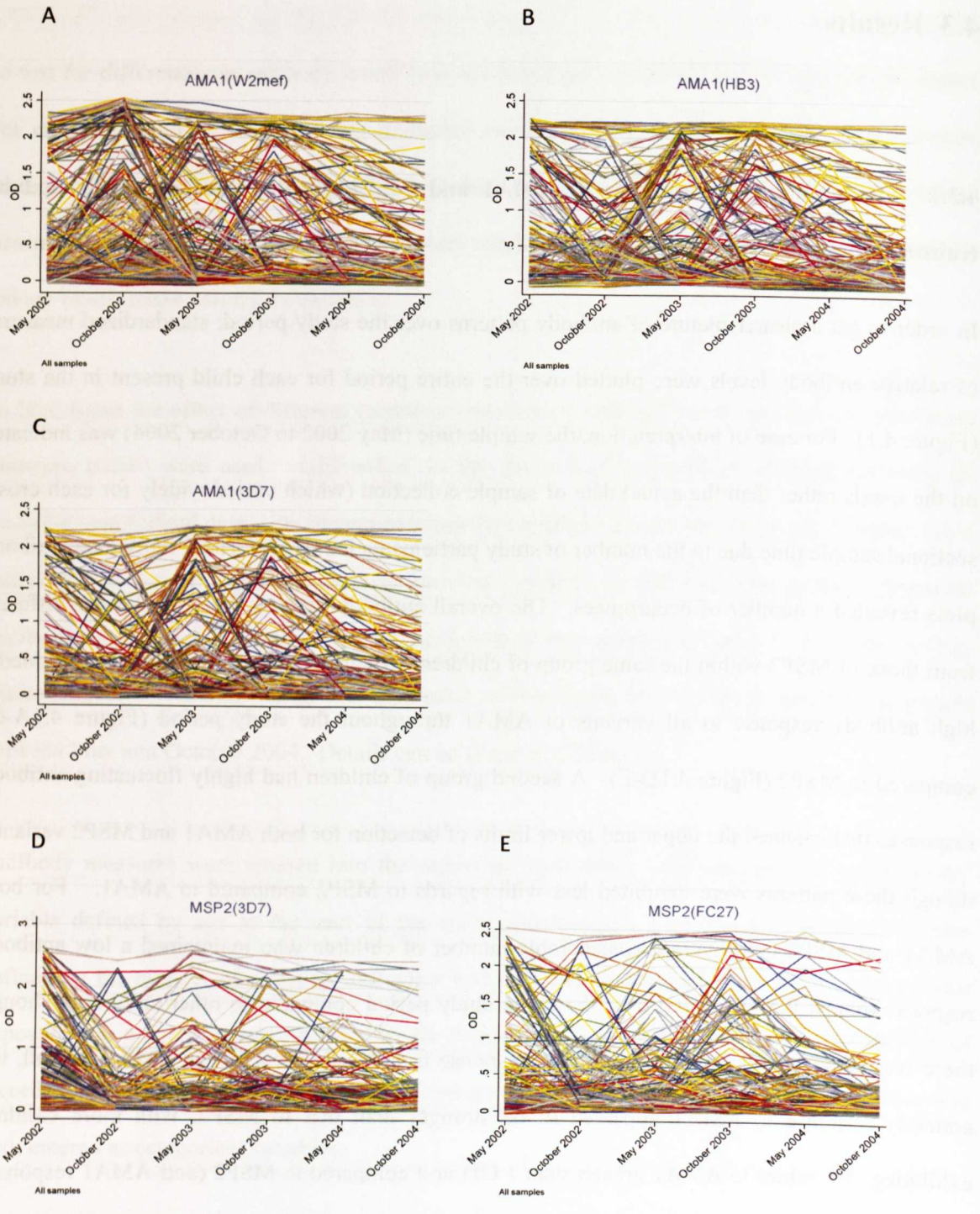

Figure 4.1 Longitudinal antibody (IgG) responses to merozoite antigens. Each line represents an individual. (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7), (E) MSP2(FC27).

To further investigate the relative stability of the antibody response to AMA1 and MSP2 over time, z-scores stratified by age and sample time were calculated for all the children present at all six sample times and each child's z-score was plotted over the study period (Figure 4.2). Both the AMA1 and MSP2 antibody data showed a considerable level of stability over time. The children

with low antibody responses (corresponding z-scores below zero) generally maintained their rank over time within the group compared to other children whose ranking changed over the study period. There were a few children with higher antibody responses who also showed a considerable level of tracking with z-scores above 1, but they were present only with regards to AMA1 (Figure 4.2 A-C). Detailed interpretation of the z-score using the graphs in Figure 4.2 is difficult due to the large number of observations in this dataset. Differences in the z-score from one cross sectional bleed to the next were used as a way to gain further insight into the relative IgG response 'position' of any one child compared to the cohort mean. The fold difference in *z*-scores was calculated and then categorised into five groups. For all the antigens tested, the greatest amount of change was up to 2-fold decline in *z*-scores between each bleed (Table 4.1). Between 40% and 70% of the children had declining IgG responses to AMA1 and MSP2 during any one interval between cross-sectional bleeds. The proportion of children who maintained their ranking ranged from 17% to 34% for MSP2 and 19% to 38% for AMA1 (Table 4.1). Generally, the largest decline in z-scores (up to 70% of children had up to a 2-fold decline in their z-scores) occurred during the May 2004 cross sectional bleed. This occurred at the same time as there was a sharp decline in the number of children with parasitaemia in the intervals between cross sectional bleeds. Between May and October 2003 37% of the children had parasitaemia compared with the following period of October 2003 to May 2004 when 12% had parasitaemia.

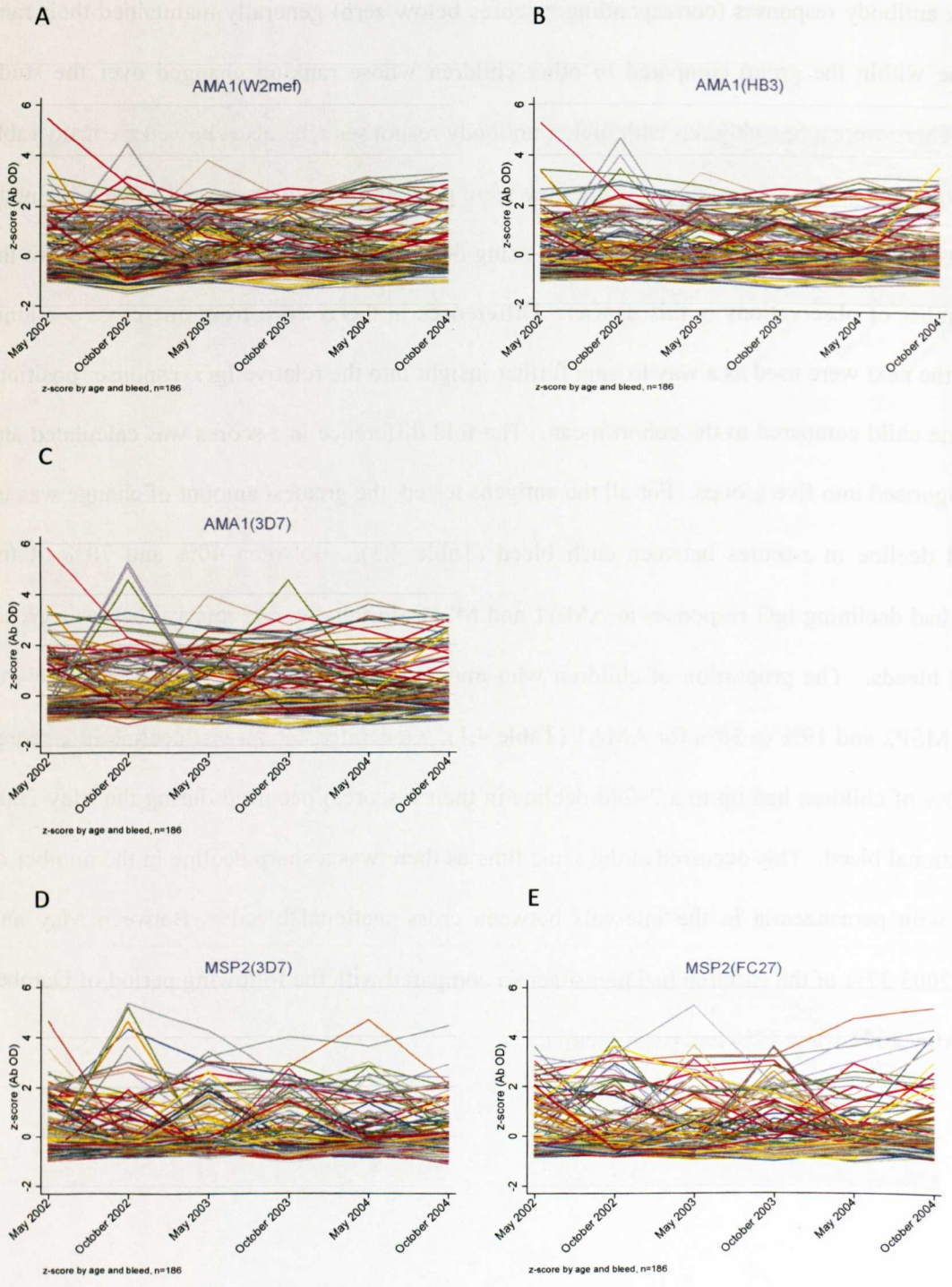

Figure 4.2 Tracking of antibody responses to merozoite antigens for children present at all six sample times. Each line represents an individual's 'response ranking' over the study period represented by their adjusted z-score (adjusted for age and sample time). (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7), (E) MSP2(FC27).

Table 4.1 Changes in z-score from one cross sectional bleed to the next over the study period.

	Change in z-score[Δ]	Anti-AMA1 and anti-MSP2 antibody response changes from previous sample point, proportion (n)				
		October 2002	May 2003	October 2003	May 2004	October 2004
AMA1(W2mef)	> 2-fold decline	8.2 (15)	5.0 (9)	6.6 (12)	2.2 (4)	3.8 (7)
	≤ 2-fold decline	40.2 (74)	67.6 (123)	47.5 (87)	66.3 (122)	50.5 (93)
	No change	38.6 (71)	19.2 (35)	38.8 (71)	25.5 (47)	38.0 (70)
	≤ 2-fold increase	7.6 (14)	1.7 (3)	3.8 (7)	2.2 (4)	6.0 (11)
	> 2-fold increase	5.4 (10)	6.6 (12)	3.3 (6)	3.8 (7)	1.6 (3)
AMA1(HB3)	> 2-fold decline	6.0 (11)	10.4 (19)	5.5 (10)	2.2 (4)	3.3 (6)
	≤ 2-fold decline	55.4 (102)	53.3 (97)	50.3 (92)	66.9 (123)	51.6 (95)
	No change	29.4 (54)	26.4 (48)	37.7 (69)	26.1 (48)	34.2 (63)
	≤ 2-fold increase	4.9 (9)	5.5 (10)	3.3 (6)	2.7 (5)	7.1 (13)
	> 2-fold increase	4.4 (8)	4.4 (8)	3.3 (6)	2.2 (4)	3.8 (7)
AMA1(3D7)	> 2-fold decline	6.0 (11)	8.8 (16)	7.1 (13)	1.1 (2)	2.2 (4)
	≤ 2-fold decline	53.3 (98)	48.4 (88)	48.6 (89)	65.2 (120)	53.8 (99)
	No change	34.2 (63)	34.1 (62)	37.2 (68)	28.3 (52)	34.8 (64)
	≤ 2-fold increase	3.3 (6)	5.5 (10)	4.4 (8)	3.3 (6)	4.9 (9)
	> 2-fold increase	3.3 (6)	3.3 (6)	2.7 (5)	2.2 (4)	4.4 (8)
MSP2(3D7)	> 2-fold decline	6.5 (12)	8.8 (16)	10.4 (19)	4.9 (9)	2.7 (5)
	≤ 2-fold decline	65.8 (121)	45.6 (83)	43.2 (79)	63.0 (116)	53.8 (99)
	No change	17.4 (32)	33.0 (60)	33.3 (61)	23.9 (44)	33.2 (61)
	≤ 2-fold increase	5.4 (10)	8.8 (16)	8.7 (16)	4.9 (9)	6.0 (11)
	> 2-fold increase	4.9 (9)	3.9 (7)	4.4 (8)	3.3 (6)	4.4 (8)
MSP2(FC27)	> 2-fold decline	5.4 (10)	8.2 (15)	6.6 (12)	2.2 (4)	7.1 (13)
	≤ 2-fold decline	51.1 (94)	52.8 (96)	51.9 (95)	70.1 (129)	50.0 (92)
	No change	34.2 (63)	27.5 (50)	27.3 (50)	23.4 (43)	31.5 (58)
	≤ 2-fold increase	5.4 (10)	7.1 (13)	8.2 (15)	2.2 (4)	6.0 (11)
	> 2-fold increase	3.8 (7)	4.4 (8)	6.0 (11)	2.2 (4)	5.4 (10)

Notes

[Δ] Difference in z-score between cross-sectional bleeds (z-score= [antibody response, OD – mean response] / standard deviation).

Table shows data for all children who were sampled at all cross sectional bleeds.

Though the IgG response to a single merozoite antigen variant from one cross-sectional bleed to the next was highly correlated (see chapter 3 for details; AMA1 $r > 0.85$ and MSP2 $r > 0.62$, all $p<0.001$ Spearman Rank); not all younger children exhibited this similarity in antibody responses to the AMA1 and MSP2 variants over the study period. Antibody profiles to AMA1 (W2mef, HB3, 23D7) and MSP2 (3D7, FC27) variants in a selection of children were plotted and only a few displayed profiles which clustered according to the antigens (Figures 4.3-4.5).

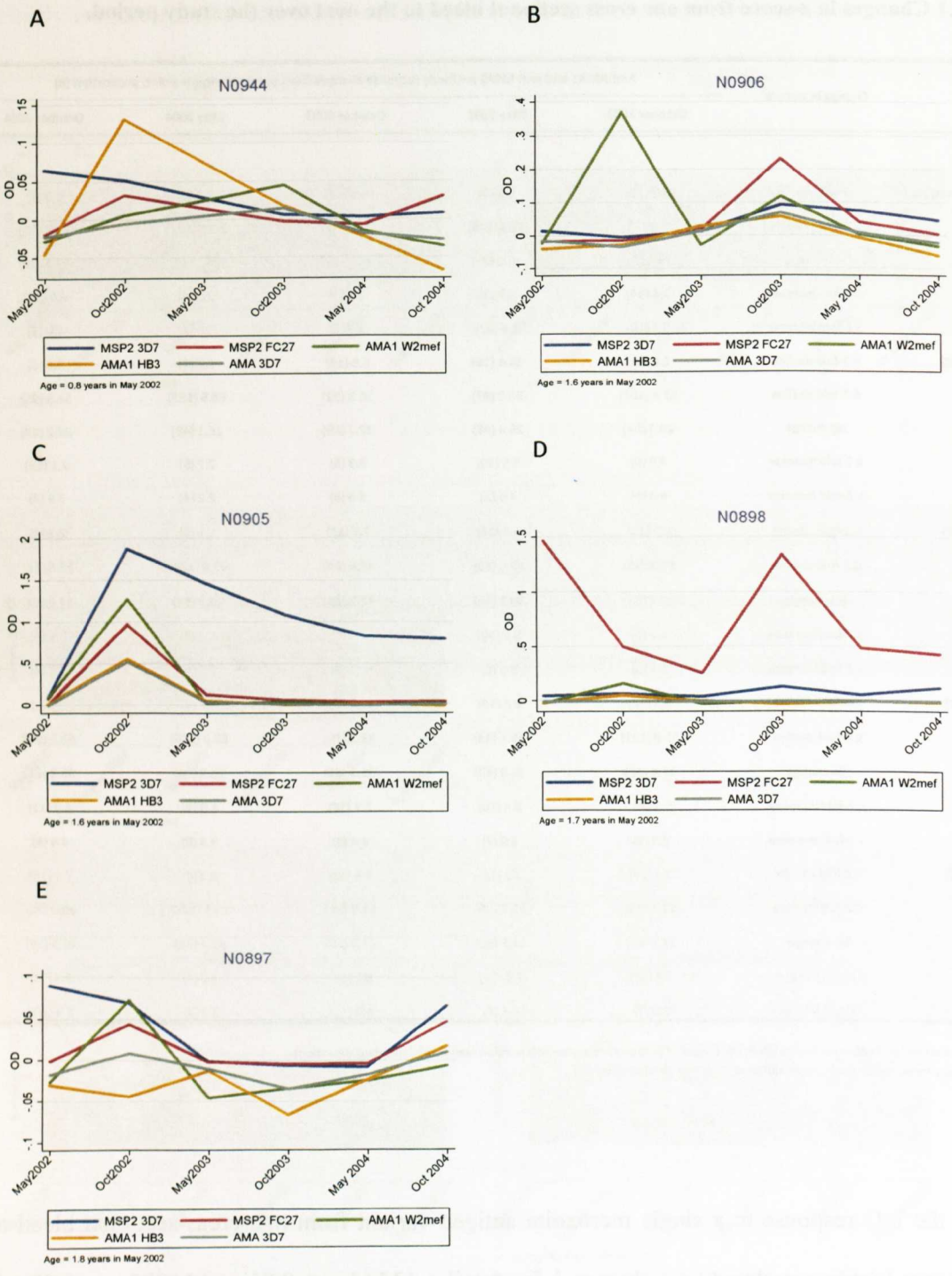

Figure 4.3 Longitudinal antibody (IgG) responses to merozoite for selected individuals between the ages of 0 and 2 years at the start of the study. Each line on the plots represents the antibody levels corresponding to a single antigen. Dark blue – MSP2(3D7), red – MSP2(FC27), green – AMA1(W2mef), yellow – AMA1(HB3), light blue – AMA1(3D7).

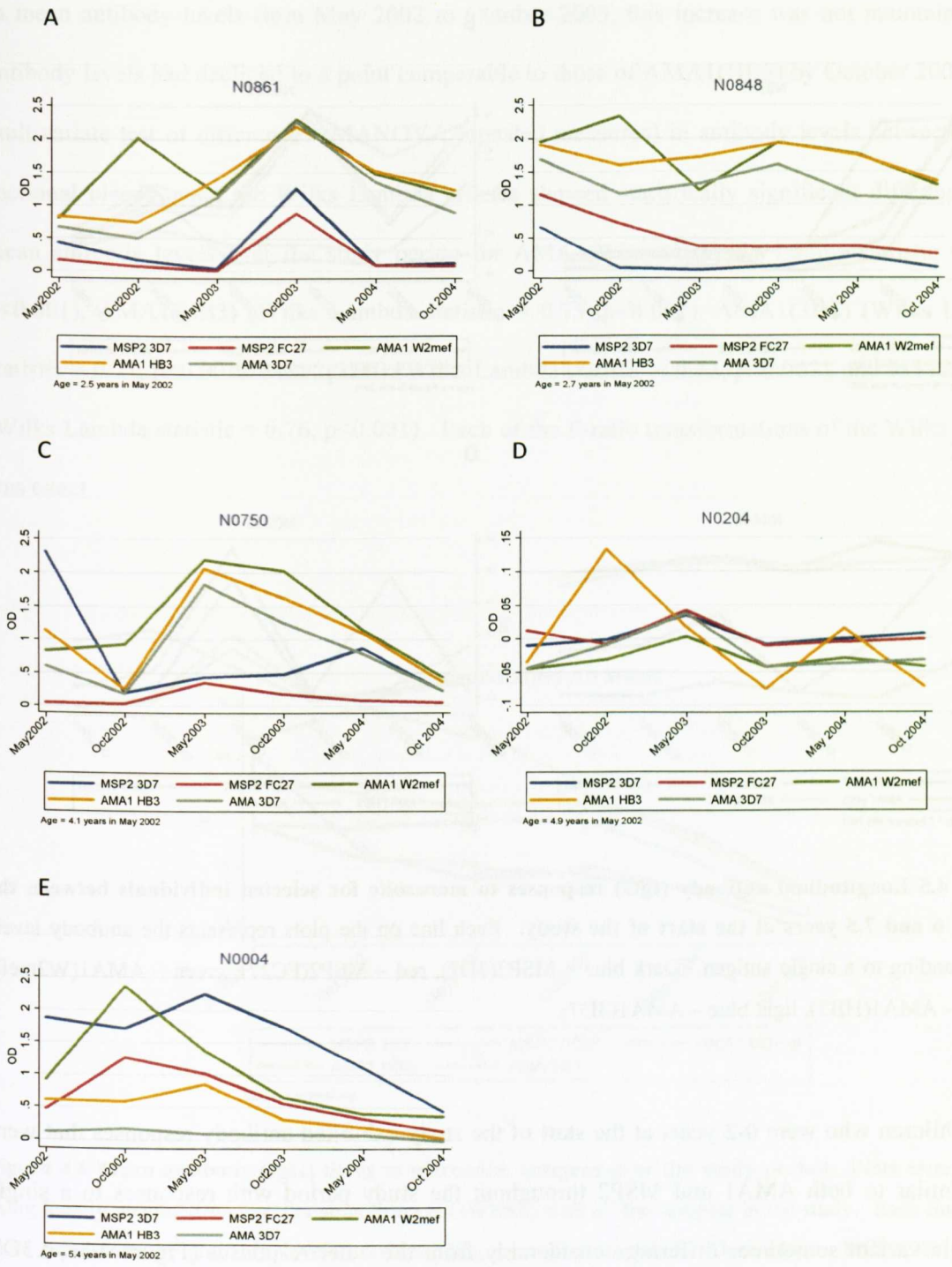

Figure 4.4 Longitudinal antibody (IgG) responses to merozoite for selected individuals between the ages of 2 and 5.5 years at the start of the study. Each line on the plots represents the antibody levels corresponding to a single antigen. Dark blue – MSP2(3D7), red – MSP2(FC27), green – AMA1(W2mef), yellow – AMA1(HB3), light blue – AMA1(3D7).

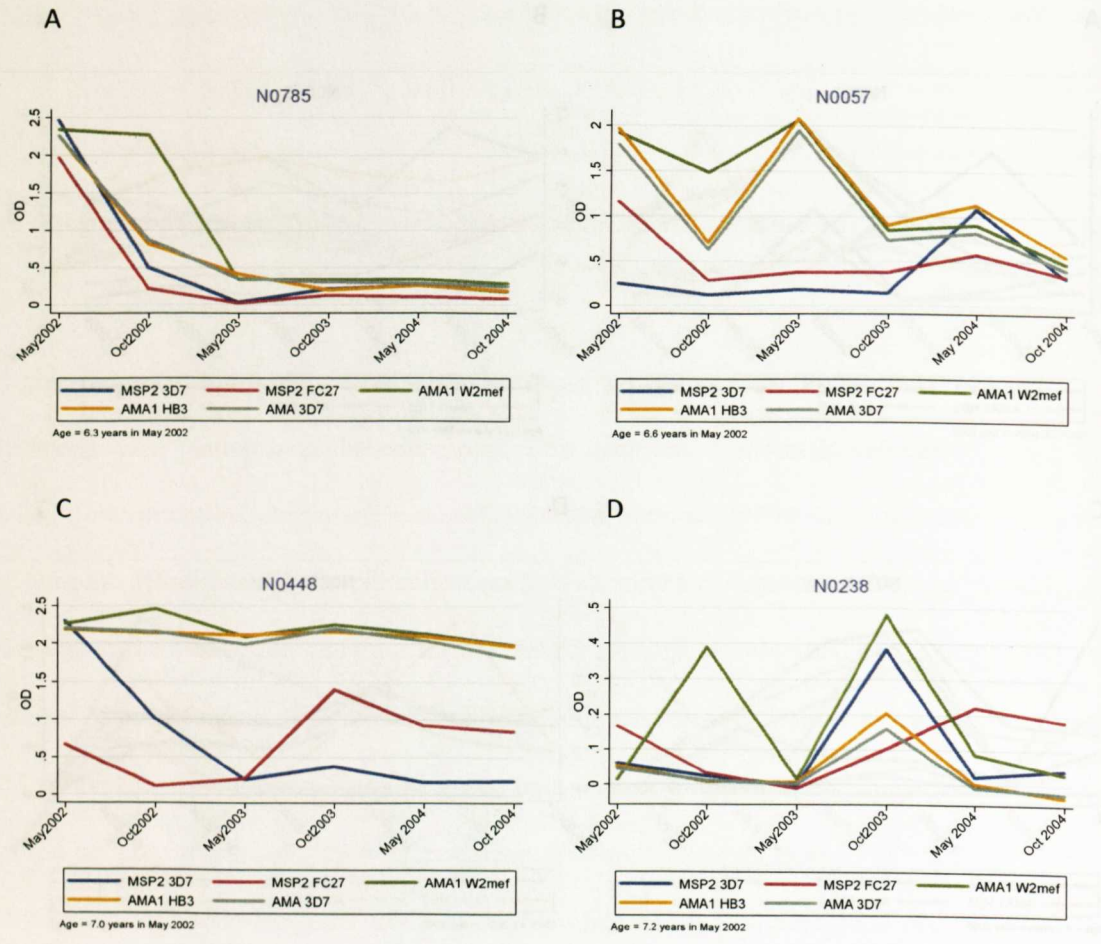

Figure 4.5 Longitudinal antibody (IgG) responses to merozoite for selected individuals between the ages of 6 and 7.5 years at the start of the study. Each line on the plots represents the antibody levels corresponding to a single antigen. Dark blue – MSP2(3D7), red – MSP2(FC27), green – AMA1(W2mef), yellow – AMA1(HB3), light blue – AMA1(3D7).

Most children who were 0-2 years at the start of the study exhibited antibody responses that were very similar to both AMA1 and MSP2 throughout the study period with responses to a single antigenic variant sometimes differing considerably from the other responses (Figure 4.3C-4.3D). Similarities in the antibody response to variants of the same antigen exhibited in concert with differences between antigens were seen in children who were older than 2 years of age at the start of the study period (Figure 4.4A-4.4C and 4.5B-4.5C). Antibody responses that were similar for all the antigens tested were also commonly seen in older children.

Even with what appear to be relatively stable antibody levels in many children when plotted individually (Figure 4.1), there was a significant overall decline in antibody levels over the study period within the study population (Figure 4.6). Though AMA1(W2mef) showed a slight increase

in mean antibody levels from May 2002 to October 2003, this increase was not maintained and antibody levels had declined to a point comparable to those of AMA1(HB3) by October 2003. The multivariate test of differences (MANOVA repeated measures) in antibody levels between cross-sectional bleeds using the Wilks Lambda criteria showed statistically significant differences in mean antibody levels over the study period for AMA1(W2mef) (Wilks Lamba statistic = 0.63, $p<0.001$), AMA1(HB3) (Wilks Lambda statistic = 0.73, $p<0.001$), AMA1(3D7) (Wilks Lambda statistic = 0.71, $p<0.001$), MSP2(3D7) (Wilks Lambda statistic = 0.73, $p<0.001$), and MSP2(FC27) (Wilks Lambda statistic = 0.76, $p<0.001$). Each of the F-ratio transformations of the Wilks criteria was exact.

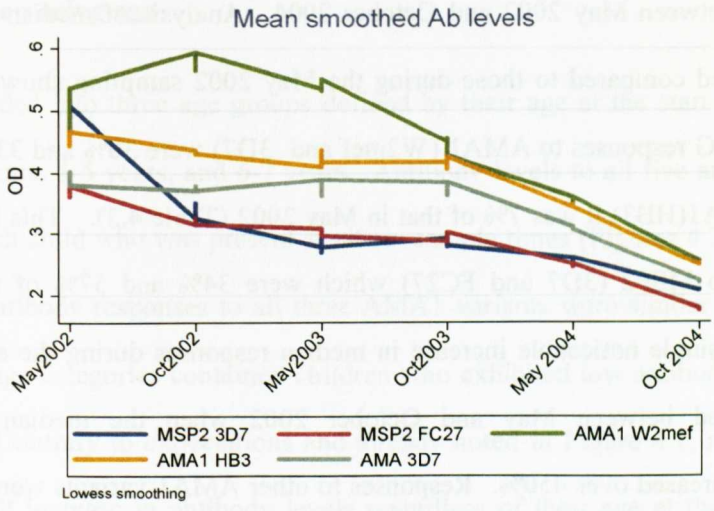

Figure 4.6 Mean antibody (IgG) titres to merozoite antigens over the study period. Plots were created using locally weighted scatter plot smoothing (LOWESS) with all the samples in the study. Each line on the plots represents the antibody levels corresponding to a single antigen. Dark blue – MSP2(3D7), red – MSP2(FC27), green – AMA1(W2mef), yellow – AMA1(HB3), light blue – AMA1(3D7).

To quantify the change in antibody levels over the study period, univariate GEE (generalised estimating equation) with exchangeable correlation structure was carried out with sample time as the dependent variable. GEE was used because it takes into account the inherent correlation (autocorrelation) between measurements carried out on the same subject repeatedly. Each successive sample time was associated with a significant decline in antibody levels of 0.06 for

AMA1(W2mef), 0.03 for AMA1 (HB3), 0.02 for AMA1(3D7) and MSP2(FC27), and 0.04 for MSP2(3D7) (all p<0.001) (Table4.2).

Table 4.2 Changes in antibody responses to AMA1 and MSP2 in Ngerenya.

	β (95% CI)[¶]	P
AMA1(W2mef)	-0.06 (-0.07, -0.05)	<0.001
AMA1(HB3)	-0.03 (-0.04, -0.02)	<0.001
AMA1(3D7)	-0.02 (-0.03, -0.01)	<0.001
MSP2(3D7)	-0.04 (-0.05, -0.03)	<0.001
MSP2(FC27)	-0.02 (-0.03, -0.01)	<0.001

Notes
Univariate generalized estimating equation (GEE) with exchangeable correlation structure.
[¶]Reduction in antibody levels at each cross sectional bleed over the entire study period.

This decline in antibody levels appears to parallel the decline in malaria transmission from 17% to 4% parasitaemia between May 2002 and October 2004. Analysis of median IgG levels at each cross sectional bleed compared to those during the May 2002 sampling showed that by October 2004 the median IgG responses to AMA1 (W2mef and 3D7) were 30% and 33% of those in May 2002, and for AMA1(HB3) it was 7% of that in May 2002 (Table 4.3). This is in contrast to the median response to MSP2 (3D7 and FC27) which were 34% and 57% of the May 02 levels respectively. The single noticeable increase in median responses during the entire period of the study was observed between May and October 2002 when the median IgG response to AMA1(W2mef) increased over 450%. Responses to other AMA1 variants were roughly the same over this period. Generally the trend was towards declining antibody responses with intervals of stability midway through the study period with most of the decline occurring over the final year of the study (Table 4.3).

Table 4.3 Changes in median IgG responses to AMA1 and MSP2 compared to the baseline cross sectional bleed.

	Proportional change in median antibody levels compared to May 2002 (n=308)				
	AMA1(W2mef)	AMA1(HB3)	AMA1(3D7)	MSP2(3D7)	MSP2(FC27)
October 2002 (294)	4.86	1.09	0.92	0.39	0.85
May 2003 (293)	0.64	1.19	0.98	0.44	0.93
October 2003 (299)	0.86	0.52	1.06	0.61	1.03
May 2004 (299)	0.52	0.56	0.61	0.35	0.79
October 2004 (302)	0.30	0.07	0.33	0.34	0.57

Notes
Difference in median IgG responses compared to responses during the May 2002 cross sectional bleed.

4.3.2 Longitudinal patterns of IgG responses to merozoite antigens do not increase in magnitude with increasing age

Children were divided into three age groups defined by their age at the start of the study period (May 2002); 0-2 years, 3-5 years, and 6-7 years. Antibody levels to all five antigens were plotted individually for each child who was present at all six sample times (Figures 4.7 and 4.8; Appendix 2 Figures 2-4). Antibody responses to all three AMA1 variants were similar in the different age groups. All three age categories contained children who exhibited low antibody levels throughout the study period. Contrary to expectations and already noted in Figure 4.1, none of the children exhibited an overall increase in antibody levels regardless of their age at the start of the study. Those children who were 3-5 years of age at the start of the study exhibited more fluctuating IgG responses to AMA1 than the 0-2 year olds (Figure 4.7B; Appendix 2, Figures 2B and 3B). Within the highest age category there appeared a group of children with consistently high antibody levels to AMA1 throughout the study period (Figure 4.7C; Appendix 2, Figures 2B and 3B). High antibody responses that were maintained over the study period were not as prevalent with regards to MSP2, even in the highest age category (Figure 4.8C; Appendix 2, Figure 4C). Most children in this age category actually exhibited very low antibody responses to both MSP2 variants. Similar to the response to AMA1, there were a number of children with widely fluctuating antibody responses to MSP2 in the 3-5 year age category, though like their older counterparts most had a low antibody response (Figure 4.8B; Appendix 2, Figure 4B).

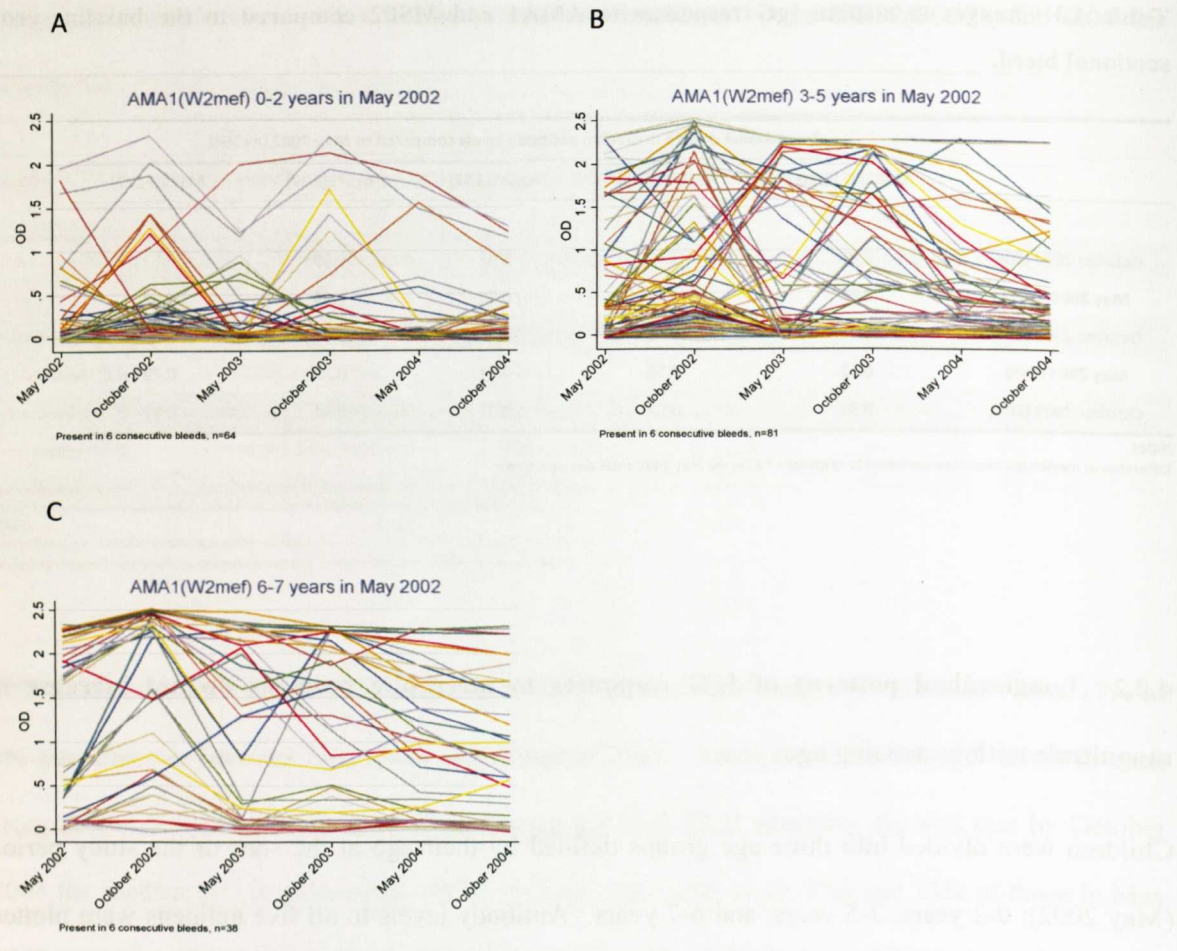

Figure 4.7 Longitudinal antibody (IgG) responses to AMA1(W2mef) for children present at all sample times. Each line represents an individual. Plots grouped by age of the child at the start of the study. (A) 0 – 2 years, (B) 3 – 5years, (C) 6 – 7 years.

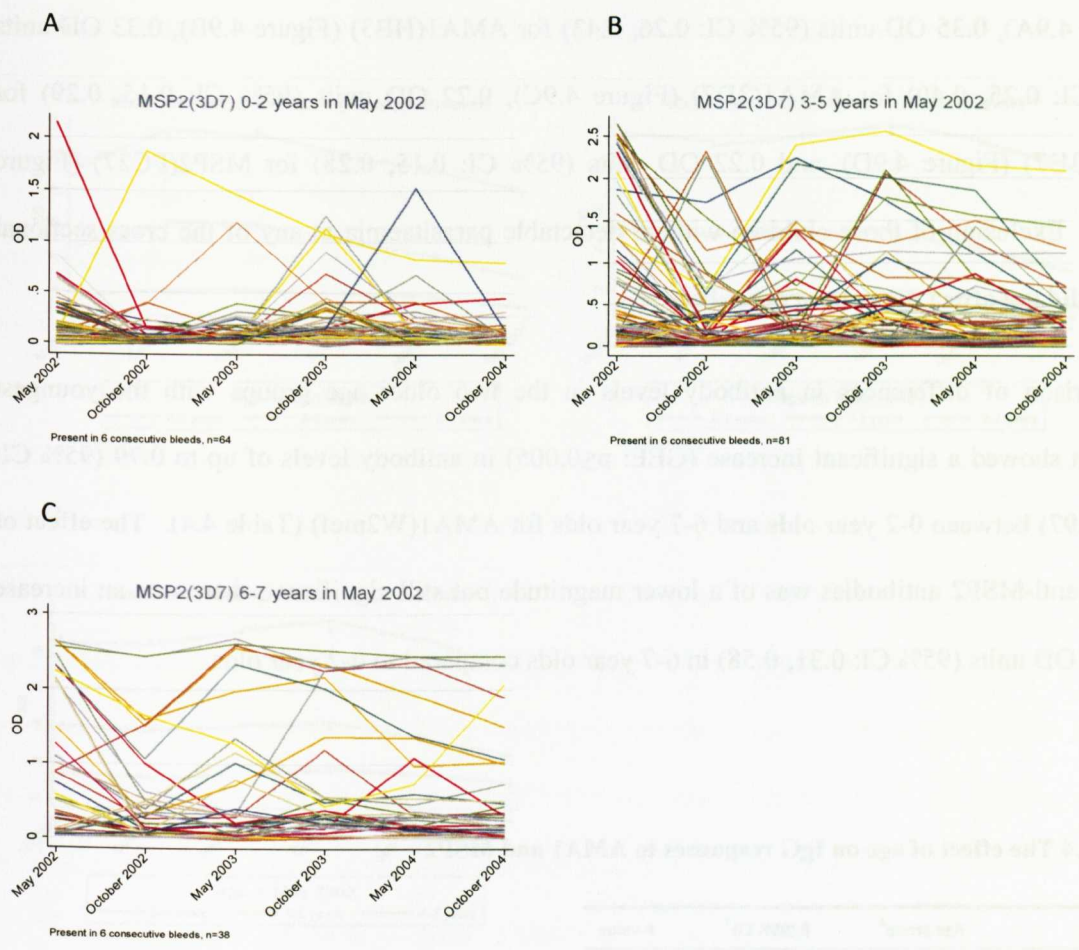

Figure 4.8 Longitudinal antibody (IgG) responses to MSP2(3D7) in children present at all sample times. Each line represents an individual. Plots grouped by age of the child at the start of the study. (A) 0 – 2 years, (B) 3 – 5years, (C) 6 – 7 years.

To better understand the effect of age on anti-AMA1 and anti-MSP2 antibodies, mean antibody levels were plotted for each of the three age categories using LOWESS smoothing techniques and differences in antibody levels throughout the study period were analysed by GEE (Figure 4.9, Table 4.4). Antibody levels to all merozoite antigens tested were highest in children who were 6-7 years at the start of the study followed by those in 3-5 year olds with the youngest children maintaining the lowest antibody levels throughout the study period. Though the children were getting older – with the 0-2 year olds being up to 4 years old in October 2004 and those who were 3-5 years in May 2002 being 7 years old at the end of the study – none of the antibody responses showed an increase with age over the study period. The increase in antibody levels with increasing age grouping was significant for all antigens (GEE: $p<0.001$). Antibody levels increased from one age group to the next on average by 0.39 OD units (95% CI: 0.30, 0.48) for AMA1(W2mef)

(Figure 4.9A), 0.35 OD units (95% CI: 0.26, 0.43) for AMA1(HB3) (Figure 4.9B), 0.33 OD units (95% CI: 0.25, 0.40) for AMA1(3D7) (Figure 4.9C), 0.22 OD units (95% CI: 0.15, 0.29) for MSP2(3D7) (Figure 4.9D), and 0.22 OD units (95% CI: 0.15, 0.28) for MSP2(FC27) (Figure 4.9E). Exclusion of those children without detectable parasitaemia at any of the cross sectional bleeds did not affect the pattern described.

Comparison of differences in antibody levels in the two older age groups with the youngest children showed a significant increase (GEE: $p \leq 0.005$) in antibody levels of up to 0.79 (95% CI: 0.62, 0.97) between 0-2 year olds and 6-7 year olds for AMA1(W2mef) (Table 4.4). The effect of age on anti-MSP2 antibodies was of a lower magnitude but still significant; there was an increase of 0.45 OD units (95% CI: 0.31, 0.58) in 6-7 year olds compared to 0-2 year olds.

Table 4.4 The effect of age on IgG responses to AMA1 and MSP2

	Age group[Δ]	β (95% CI)[ǂ]	*P*-value
AMA1(W2mef)	3-5 years	0.33 (0.18, 0.48)	<0.001
	6-7 years	0.79 (0.62, 0.97)	<0.001
AMA1(HB3)	3-5 years	0.29 (0.15, 0.43)	<0.001
	6-7 years	0.71 (0.54, 0.88)	<0.001
AMA1(3D7)	3-5 years	0.23 (0.1, 0.36)	0.001
	6-7 years	0.68 (0.52, 0.84)	<0.001
MSP2(3D7)	3-5 years	0.22 (0.11, 0.33)	<0.001
	6-7 years	0.44 (0.31, 0.57)	<0.001
MSP2(FC27)	3-5 years	0.16 (0.05, 0.27)	0.005
	6-7 years	0.45 (0.31, 0.58)	<0.001

Notes

Univariate generalized estimating equation (GEE) with exchangeable correlation structure (n=299).

[Δ] Age group during May 2002 sample time.

[ǂ] GEE regression coefficients indicating reduction/increase in the antibody response compared to children age 0-2 years.

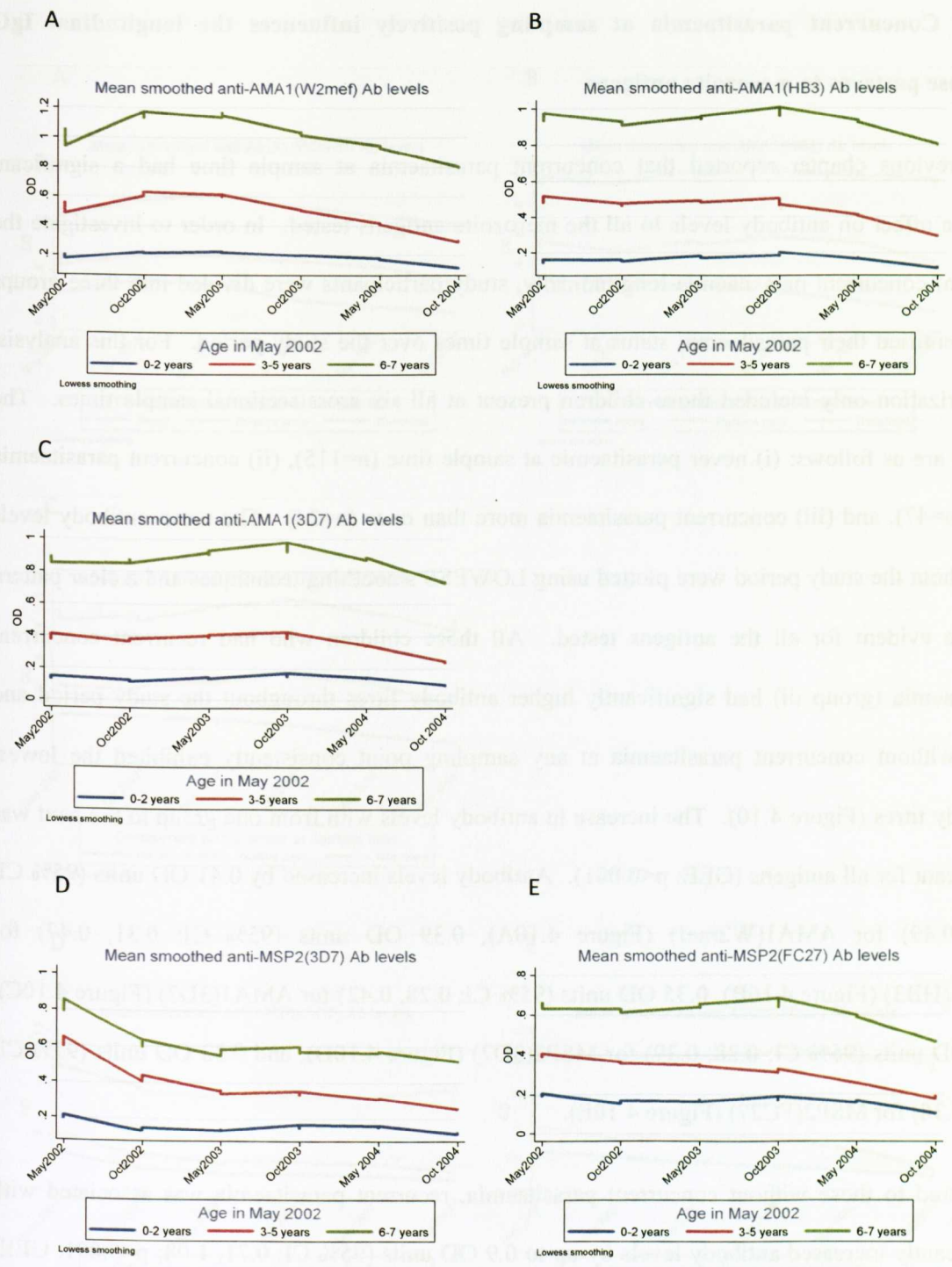

Figure 4.9 Mean antibody (IgG) titres to merozoite antigens over the study period. Plots were created using locally weighted scatterplot smoothing (LOWESS) with all the samples in the study. Each line on the plots represents the antibody levels corresponding to an age grouping at the start of the study; 0-2 years (blue), 3-5 years (red), 6-7 years (green). (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7), (E) MSP2(FC27).

4.3.3 Concurrent parasitaemia at sampling positively influences the longitudinal IgG response patterns to merozoite antigens

The previous chapter reported that concurrent parasitaemia at sample time had a significant positive effect on antibody levels to all the merozoite antigens tested. In order to investigate the effect of concurrent parasitaemia longitudinally, study participants were divided into three groups that identified their parasitaemic status at sample times over the study period. For this analysis, categorization only included those children present at all six cross-sectional sample times. The groups are as follows: (i) never parasitaemic at sample time (n=115), (ii) concurrent parasitaemia once (n=47), and (iii) concurrent parasitaemia more than once (n=24). The mean antibody levels throughout the study period were plotted using LOWESS smoothing techniques and a clear pattern became evident for all the antigens tested. All those children who had recurrent concurrent parasitaemia (group iii) had significantly higher antibody titres throughout the study period and those without concurrent parasitaemia at any sampling point consistently exhibited the lowest antibody titres (Figure 4.10). The increase in antibody levels with from one group to the next was significant for all antigens (GEE: $p<0.001$). Antibody levels increased by 0.41 OD units (95% CI: 0.33, 0.49) for AMA1(W2mef) (Figure 4.10A), 0.39 OD units (95% CI: 0.31, 0.47) for AMA1(HB3) (Figure 4.10B), 0.35 OD units (95% CI: 0.28, 0.42) for AMA1(3D7) (Figure 4.10C), 0.34 OD units (95% CI: 0.28, 0.39) for MSP2(3D7) (Figure 4.10D), and 0.33 OD units (95% CI: 0.27, 0.38) for MSP2(FC27) (Figure 4.10E).

Compared to those without concurrent parasitaemia, recurrent parasitaemia was associated with significantly increased antibody levels by up to 0.9 OD units (95% CI: 0.71, 1.08; $p<0.001$, GEE) for AMA1(W2mef) and 0.82 OD units (95% CI: 0.70, 0.93; $p<0.001$, GEE) for MSP2(3D7) (Table 4.5).

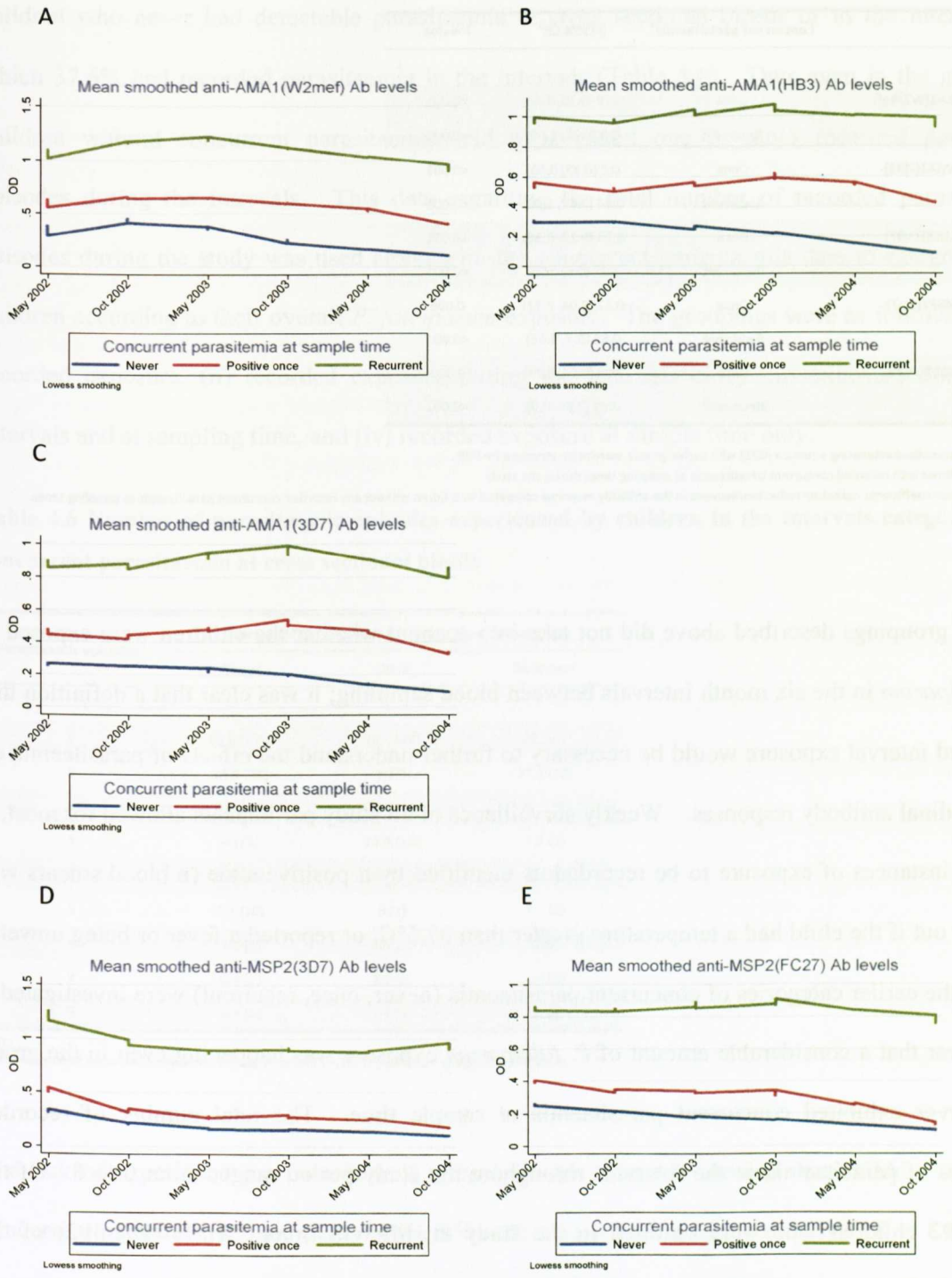

Figure 4.10 Mean antibody (IgG) titres to merozoite antigens over the study period. Plots were created using locally weighted scatterplot smoothing (LOWESS) with all the samples in the study. Each line on the plots represents the antibody levels corresponding to the number of times concurrent parasitaemia was recorded at sample time; never parasitaemic (blue), concurrent parasitaemia at one sample time (red), concurrent parasitaemia more than once (green). (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7), (E) MSP2(FC27).

Table 4.5 The effect of concurrent parasitaemia on IgG responses to AMA1 and MSP2

	Concurrent parasitaemia[Δ]	β (95% CI)[ǂ]	*P*-value
AMA1(W2mef)	Once	0.29 (0.15, 0.43)	<0.001
	Recurrent	0.9 (0.71, 1.08)	<0.001
AMA1(HB3)	Once	0.3 (0.17, 0.43)	<0.001
	Recurrent	0.84 (0.67, 1.02)	<0.001
AMA1(3D7)	Once	0.25 (0.12, 0.38)	<0.001
	Recurrent	0.77 (0.6, 0.93)	<0.001
MSP2(3D7)	Once	0.12 (0.04, 0.21)	0.006
	Recurrent	0.82 (0.7, 0.93)	<0.001
MSP2(FC27)	Once	0.14 (0.05, 0.24)	0.003
	Recurrent	0.78 (0.66, 0.9)	<0.001

Notes

Univariate generalized estimating equation (GEE) with exchangeable correlation structure (n=398).

[Δ]Number of times with recorded concurrent parasitaemia at sampling times during the study.

[ǂ]GEE regression coefficients indicating reduction/increase in the antibody response compared to children without any recorded concurrent parasitaemia at sampling times.

As the groupings described above did not take into account whether the children were exposed to *P. falciparum* in the six month intervals between blood sampling; it was clear that a definition that included interval exposure would be necessary to further understand the effects of parasitaemia on longitudinal antibody responses. Weekly surveillance of all study participants allowed for most, if not all instances of exposure to be recorded as identified by a positive slide (a blood smears was carried out if the child had a temperature greater than 37.5°C, or reported a fever or being unwell). When the earlier categories of concurrent parasitaemia (never, once, recurrent) were investigated it was clear that a considerable amount of *P. falciparum* exposure was happening even in the group that never exhibited concurrent parasitaemia at sample time. The total number of recorded episodes of parasitaemia in the intervals throughout the study period ranged from 0 to 8. Of the total 392 children who were enrolled in the study at different times, 51.3% (201/392) of the children did not have any recorded parasitaemia in the intervals between sampling times throughout the study period (Table 4.6). Of these children 85.1% (171/201) were categorized as never having any concurrent parasitaemia at sample time. 9% (18/201) of these children had concurrent parasitaemia only once and 6% (12/201) of those who had recurrent concurrent parasitaemia didn't experience any recorded parasitaemia in the intervals. Within each of these three categories the proportion of children with one or more parasitaemic episodes in the intervals was high in the group of children with concurrent parasitaemia once (76.9%) and those with

recurrent concurrent parasitaemia (70%). These proportions contrasted with that in the group of children who never had detectable parasitaemia at cross sectional bleeds or in the intervals of which 37.6% had recorded parasitaemia in the intervals (Table 4.6). Thus even in the group of children without concurrent parasitaemia had experienced one or more recorded parsitemic episodes during the intervals. This data regarding the total number of recorded parasitaemic episodes during the study was used along with the concurrent parasitaemia data to categorise the children according to their overall *P. falciparum* exposure. The groupings were as follows: (i) No recorded exposure, (ii) recorded exposure during the intervals only, (iii) exposure during the intervals and at sampling time, and (iv) recorded exposure at sample time only.

Table 4.6 Number of parasitaemic episodes experienced by children in the intervals categorised by concurrent parasitaemia at cross sectional bleeds

Parasitaemic episodes[Δ]	Concurrent parasitaemia at sample time, percent (n)		
	Never	Once	Recurrent
0	62.4 (171)	23.1 (18)	30 (12)
1	13.9 (38)	9 (7)	37.5 (15)
2	8.4 (23)	18 (14)	12.5 (5)
3	4 (11)	15.4 (12)	7.5 (3)
4	5.5 (15)	16.7 (13)	5 (2)
5	3.7 (10)	9 (7)	2.5 (1)
6	1.1 (3)	5.1 (4)	0 (0)
7	0.4 (1)	2.6 (2)	2.5 (1)
8	0.7 (2)	1.3 (1)	2.5 (1)

Notes
[Δ] Total number of parasitaemic episodes during the entire study recorded during weekly surveillance.

The risk of experiencing a parasitaemic episode in the intervals showed that children who had concurrent parasitaemia once (RR = 1.95, 95% CI: 1.65 – 2.30) and recurrently (RR = 1.51, 95% CI: 1.20 – 1.90) were at a significantly higher risk ($p<0.001$) of parasitaemia throughout the study period than those who did not have any concurrent parasitaemia at time of sampling. When median IgG responses at a single bleed (October 2002) were analysed by both age and the number of times they had detectable concurrent parasitaemia at sampling, those with concurrent parasitaemia once or recurrently showed an increase in antibody responses with age (Appendix 2, Table 4 and 5). Median IgG responses to AMA1 and MSP2 were higher in the oldest children with recurrent parasitaemia when compared to their peers who had only one instance of concurrent parasitaemia

($p \leq 0.05$, Wilcoxon rank sum) (Appendix 2, Table 4 and 5). These differences were only apparent when all the samples were analysed together only when children who were aparasitaemic during the October 2002 cross sectional bleed were considered.

Longitudinal plots of mean antibody responses within these groupings showed that those children without exposure during the study period had consistently low antibody levels to all the merozoite antigens tested (Figure 4.11). Those with exposure recorded during the intervals only had slightly higher antibody titres than those without any recorded exposure. The highest antibody responses to both AMA1 and MSP2 were seen in children who were parasitaemic only at sample time (Figure 4.11). Investigation into the difference in antibody levels throughout the study period by GEE in different exposure categories showed that antibody levels to both MSP2(3D7), AMA1(W2mef) and AMA1(3D7) were no different in the group without exposure and the group with exposure only during the intervals (Table 4.7). Only in the case of anti-AMA1(HB3) antibodies was a significant increase of 0.13 OD units (95% CI:0, 0.26; p=0.05) over the study period in the group with interval exposure only compared to that without any recorded exposure. Antibody responses to all the antigens were significantly higher ($p<0.001$) over the study period when individuals had concurrent parasitaemia regardless of their exposure to *P. falciparum* in the intervals (Table 4.7).

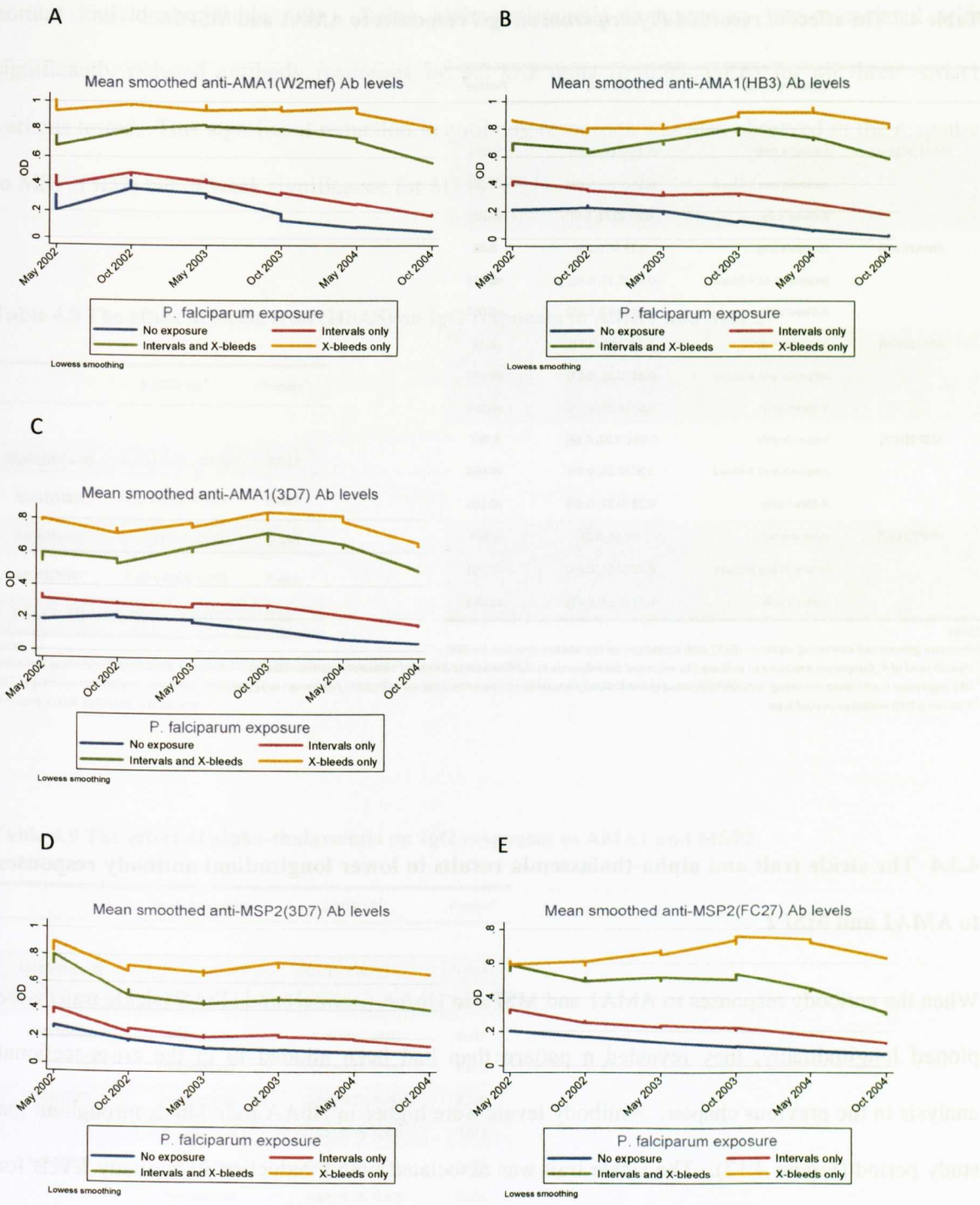

Figure 4.11 Mean antibody (IgG) titres to merozoite antigens over the study period. Plots were created using locally weighted scatterplot smoothing (LOWESS) with all the samples in the study. Each line on the plots represents the antibody levels with regards overall recorded *P. falciparum* exposure throughout the study; no exposure (blue), exposure during the intervals only (red), exposure during the intervals and at sample time (green), exposure at sample time only (yellow). (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7), (E) MSP2(FC27).

Table 4.7 The effect of recorded *P. falciparum* on IgG responses to AMA1 and MSP2

	Exposure Level[1]	β (95% CI)[‡]	*P*-value[Δ]
AMA1(W2mef)	Intervals only	0.1 (-0.04, 0.25)	0.152
	Intervals and X-Bleed	0.51 (0.36, 0.66)	**<0.001**
	X-Bleed only	0.67 (0.43, 0.9)	**<0.001**
AMA1(HB3)	Intervals only	0.13 (0, 0.26)	**0.05**
	Intervals and X-Bleed	0.51 (0.37, 0.65)	**<0.001**
	X-Bleed only	0.64 (0.42, 0.86)	**<0.001**
AMA1(3D7)	Intervals only	0.1 (-0.03, 0.22)	**0.13**
	Intervals and X-Bleed	0.44 (0.31, 0.57)	**<0.001**
	X-Bleed only	0.57 (0.36, 0.78)	**<0.001**
MSP2(3D7)	Intervals only	0.08 (-0.02, 0.18)	0.097
	Intervals and X-Bleed	0.36 (0.26, 0.46)	**<0.001**
	X-Bleed only	0.53 (0.37, 0.69)	**<0.001**
MSP2(FC27)	Intervals only	0.1 (0, 0.2)	0.054
	Intervals and X-Bleed	0.37 (0.27, 0.48)	**<0.001**
	X-Bleed only	0.51 (0.34, 0.67)	**<0.001**

Notes

Univariate generalized estimating equation (GEE) with exchangeable correlation structure (n=389).

[1] Overall level of *P. falciparum* exposure as indicated by recorded parasitaemia in the intervals and at cross sectional bleeds (X-bleeds).

[‡] GEE regression coefficients indicating reduction/increase in the antibody response compared to children without *P. falciparum* exposure.

[Δ] *P*-values ≤ 0.05 indicated in bold type.

4.3.4 The sickle trait and alpha-thalassemia results in lower longitudinal antibody responses to AMA1 and MSP2

When the antibody responses to AMA1 and MSP2 in HbAA (normal) and HbAS (sickle trait) were plotted longitudinally, they revealed a pattern than had been alluded to in the cross-sectional analysis in the previous chapter. Antibody levels were higher in HbAA individuals throughout the study period (Figure 4.12). The sickle trait was associated with a reduction in antibody levels for all the antigens tested of approximately 0.1 OD units for MSP2 variants and 0.2 OD units for AMA1 variants; these differences were statistically significant only for AMA1 (Table 4.8).

Longitudinal plots of antibody levels in normal, heterozygous, and homozygous alpha-thalassemic individuals revealed that the normal and heterozygous children had longitudinal antibody levels that were very similar (Figure 4.13). In comparison, children who were homozygous for alpha-thalassemia had visibly lowered antibody levels throughout the study period which declined in concert with the reduction in malaria transmission over the study period. Analysis by GEE showed that there was no significant difference in antibody levels between the heterozygotes and

normal individuals (Table 4.9). Being alpha-thalassemia homozygous was associated with significantly reduced antibody responses by 0.2 OD units ($p<0.05$, GEE) for all three AMA1 variants tested. This significant reduction in antibody responses was also observed in the response to MSP2(3D7) but of weak significance for MSP(FC27).

Table 4.8 The effect of sickle trait (HbAS) on IgG responses to AMA1 and MSP2

	β (95% CI)[‡]	*P*-value[Δ]
AMA1(W2mef)	-0.23 (-0.43, -0.04)	**0.017**
AMA1(HB3)	-0.2 (-0.38, -0.02)	**0.027**
AMA1(3D7)	-0.19 (-0.36, -0.02)	**0.031**
MSP2(3D7)	-0.05 (-0.19, 0.08)	0.452
MSP2(FC27)	-0.1 (-0.24, 0.03)	0.132

Notes

Univariate generalized estimating equation (GEE) with exchangeable correlation structure (n=355).

[‡]GEE regression coefficients indicating reduction/increase in the antibody response compared to children without the sickle trait (HbAA).

[Δ]*P*-values ≤ 0.05 indicated in bold type.

Table 4.9 The effect of alpha-thalassemia on IgG responses to AMA1 and MSP2

	Alpha-thalassemia	β (95% CI)[‡]	*P*-value[Δ]
AMA1(W2mef)	Heterozygous	-0.03 (-0.18, 0.12)	0.656
	Homozygous	-0.24 (-0.43, -0.04)	**0.016**
AMA1(HB3)	Heterozygous	-0.06 (-0.2, 0.08)	0.377
	Homozygous	-0.23 (-0.42, -0.05)	**0.012**
AMA1(3D7)	Heterozygous	-0.04 (-0.17, 0.09)	0.565
	Homozygous	-0.21 (-0.38, -0.04)	**0.017**
MSP2(3D7)	Heterozygous	-0.06 (-0.16, 0.05)	0.276
	Homozygous	-0.16 (-0.29, -0.02)	**0.024**
MSP2(FC27)	Heterozygous	-0.01 (-0.12, 0.09)	0.832
	Homozygous	-0.13 (-0.26, 0.01)	0.062

Notes

Univariate generalized estimating equation (GEE) with exchangeable correlation structure (n=349).

[‡]GEE regression coefficients indicating reduction/increase in the antibody response compared to normal children.

[Δ]*P*-values ≤ 0.05 indicated in bold type.

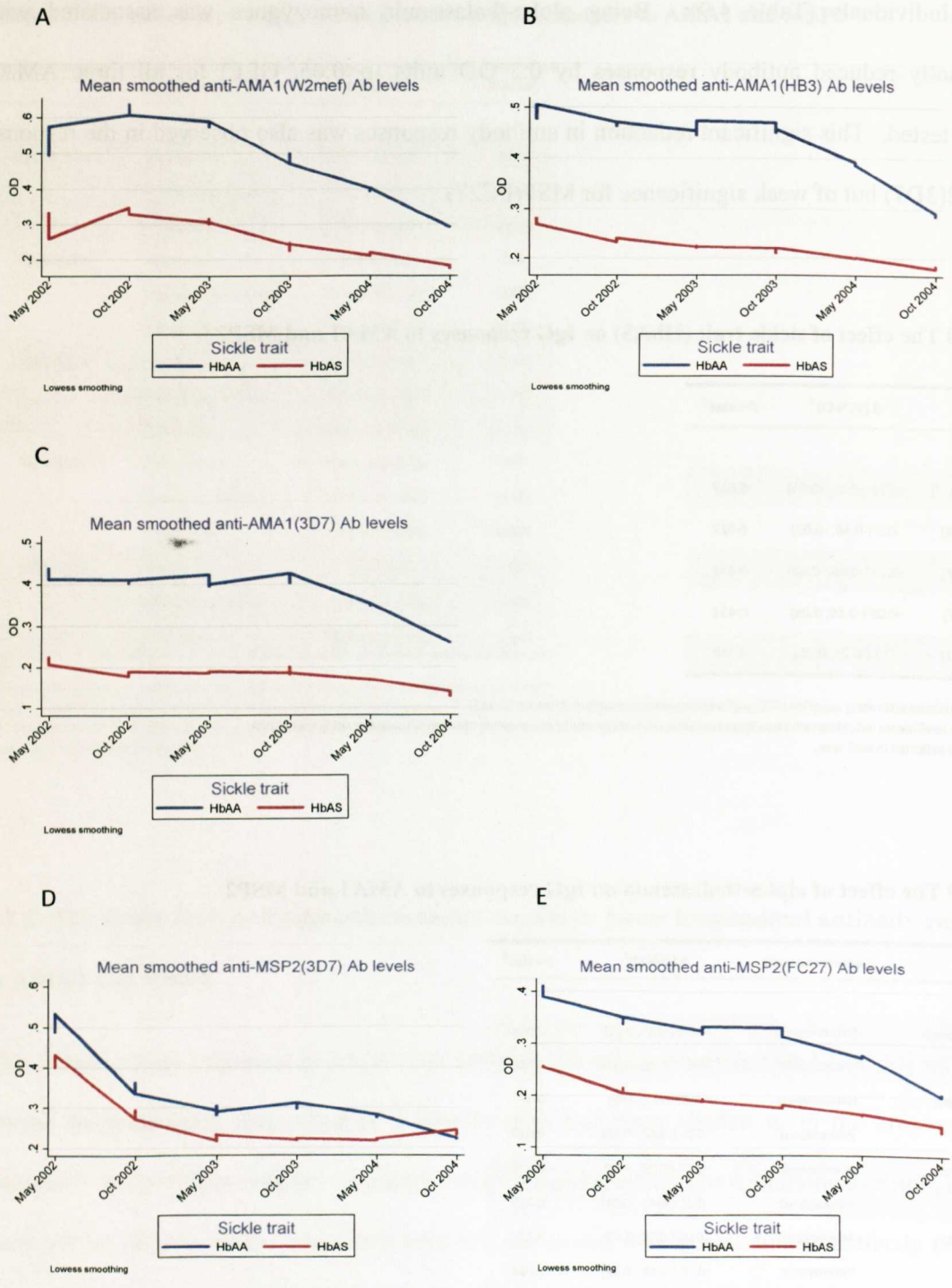

Figure 4.12 Mean antibody (IgG) titres to merozoite antigens over the study period in HbAA and HbAS chidren. Plots were created using locally weighted scatterplot smoothing (LOWESS) with all the samples in the study. Each line on the plots represents the antibody levels with regards to sickle trait; normal (blue), and sickle trait (red). (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7), (E) MSP2(FC27).

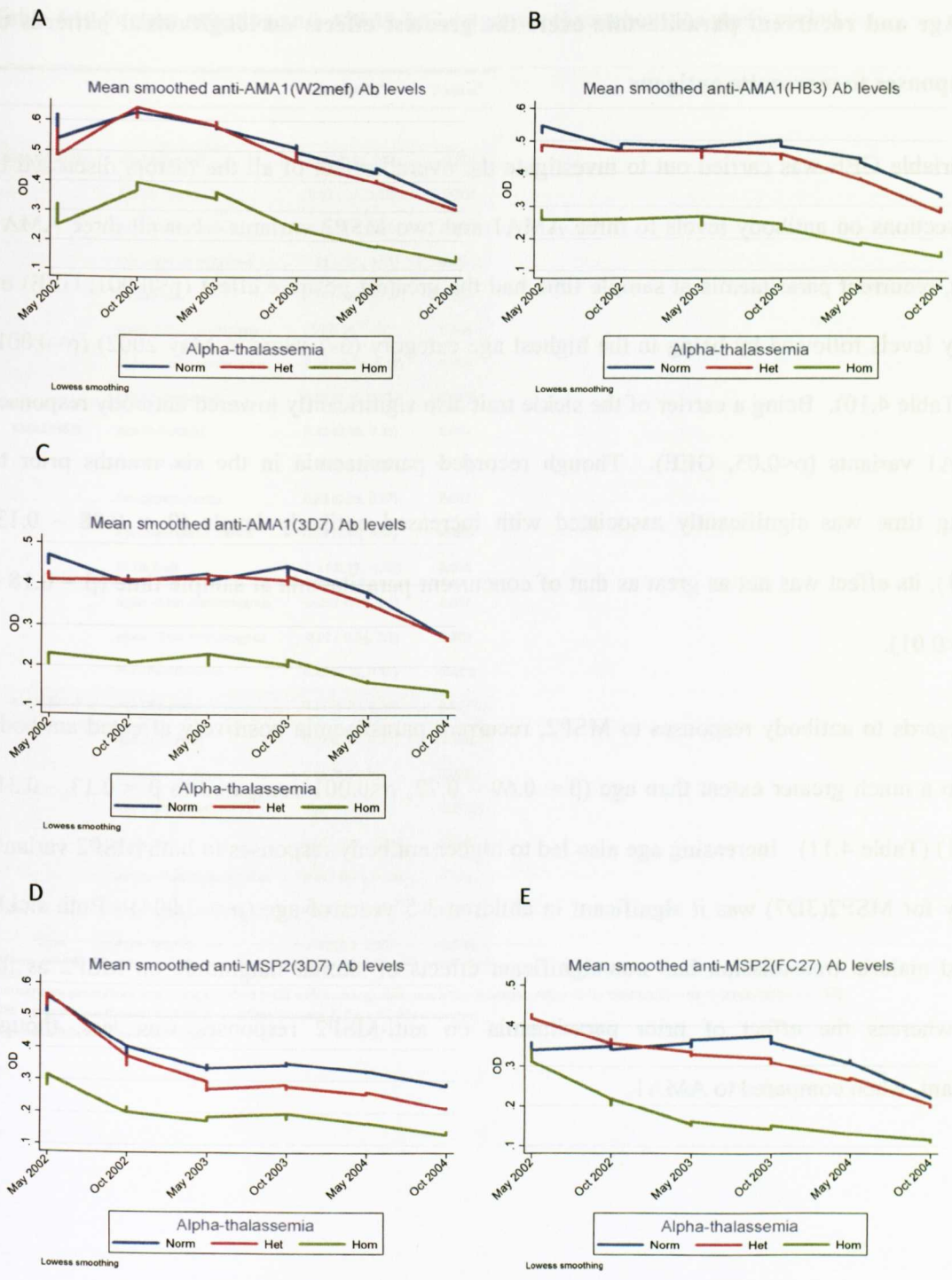

Figure 4.13 Mean antibody (IgG) titres to merozoite antigens over the study period in children with and without alpha-thalassemia. Plots were created using locally weighted scatter plot smoothing (LOWESS) with all the samples in the study. Each line on the plots represents the antibody levels with regards to alpha-thalassemia; normal (blue), heterozygous (red), homozygous (green). (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7), (E) MSP2(FC27).

4.3.5 Age and recurrent parasitaemia exert the greatest effects on longitudinal patterns of IgG responses to merozoite antigens

Multivariable GEE was carried out to investigate the overall effect of all the factors discussed in earlier sections on antibody levels to three AMA1 and two MSP2 variants. For all three AMA1 variants, recurrent parasitaemia at sample time had the greatest positive effect ($p<0.001$, GEE) on antibody levels followed by being in the highest age category (6-7 years in May 2002) ($p<0.001$, GEE) (Table 4.10). Being a carrier of the sickle trait also significantly lowered antibody responses to AMA1 variants ($p<0.05$, GEE). Though recorded parasitaemia in the six months prior to sampling time was significantly associated with increased antibody levels ($\beta = 0.08 - 0.13$; $p<0.001$), its effect was not as great as that of concurrent parasitaemia at sample time ($\beta = 0.18 - 0.71$; $p<0.01$).

With regards to antibody responses to MSP2, recurrent parasitaemia positively affected antibody levels to a much greater extent than age ($\beta = 0.69 - 0.72$; $p<0.001$ compared to $\beta = 0.13 - 0.31$; $p<0.001$) (Table 4.11). Increasing age also led to higher antibody responses to both MSP2 variants but only for MSP2(3D7) was it significant in children 3-5 years of age ($p = 0.004$). Both sickle trait and malaria transmission had non-significant effects of similar magnitude on MSP2 as for AMA whereas the effect of prior parasitaemia on anti-MSP2 responses was less, though significant, when compared to AMA1.

Table 4.10 Factors affecting anti-AMA1 IgG responses throughout the study period

	Factor	β (95% CI)[‡]	*P*-value[Δ]
AMA1(W2mef)	Age (3-5 years)	0.26 (0.12, 0.4)	**<0.001**
	Age (6-7 years)	0.67 (0.5, 0.83)	**<0.001**
	Parasitaemic once	0.25 (0.1, 0.39)	**0.001**
	Recurrent parasitaemia	0.71 (0.52, 0.91)	**<0.001**
	Sickle Trait	-0.25 (-0.43, -0.07)	**0.006**
	alpha –Thal. Heterozygous	0 (-0.14, 0.14)	0.968
	alpha –Thal. Homozygous	-0.06 (-0.24, 0.12)	0.496
	Prior Parasitaemia	0.08 (0.05, 0.11)	**<0.001**
AMA1(HB3)	Age (3-5 years)	0.22 (0.09, 0.35)	**0.001**
	Age (6-7 years)	0.59 (0.43, 0.75)	**<0.001**
	Parasitaemic once	0.23 (0.09, 0.37)	**0.001**
	Recurrent parasitaemia	0.64 (0.46, 0.82)	**<0.001**
	Sickle Trait	-0.2 (-0.37, -0.03)	**0.019**
	alpha –Thal. Heterozygous	-0.03 (-0.16, 0.1)	0.657
	alpha –Thal. Homozygous	-0.07 (-0.24, 0.1)	0.409
	Prior Parasitaemia	0.13 (0.11, 0.15)	**<0.001**
AMA1(3D7)	Age (3-5 years)	0.17 (0.04, 0.29)	**0.007**
	Age (6-7 years)	0.57 (0.42, 0.72)	**<0.001**
	Parasitaemic once	0.18 (0.04, 0.31)	**0.008**
	Recurrent parasitaemia	0.57 (0.4, 0.74)	**<0.001**
	Sickle Trait	-0.18 (-0.34, -0.02)	**0.026**
	alpha –Thal. Heterozygous	-0.01 (-0.13, 0.12)	0.896
	alpha –Thal. Homozygous	-0.06 (-0.23, 0.1)	0.437
	Prior Parasitaemia	0.12 (0.1, 0.15)	**<0.001**

Notes

Multivariable generalized estimating equation (GEE) with exchangeable correlation structure, adjusted for changes in malaria transmission (n=287).

[‡] GEE regression coefficients indicating reduction/increase in the antibody response.

[Δ] *P*-values ≤ 0.05 indicated in bold type.

Table 4.11 Factors affecting anti-MSP2 IgG responses throughout the study period

	Factor	β (95% CI)[‡]	*P*-value[Δ]
MSP2(3D7)	Age (3-5 years)	0.13 (0.04, 0.22)	**0.004**
	Age (6-7 years)	0.3 (0.19, 0.41)	**<0.001**
	Parasitaemic once	0.08 (-0.02, 0.17)	0.109
	Recurrent parasitaemia	0.72 (0.59, 0.84)	**<0.001**
	Sickle Trait	-0.07 (-0.19, 0.05)	0.246
	alpha –Thal. Heterozygous	0.02 (-0.07, 0.11)	0.626
	alpha –Thal. Homozygous	-0.02 (-0.14, 0.1)	0.792
	Prior Parasitaemia	0.08 (0.06, 0.11)	**<0.001**
MSP2(FC27)	Age (3-5 years)	0.07 (-0.03, 0.17)	0.158
	Age (6-7 years)	0.31 (0.19, 0.43)	**<0.001**
	Parasitaemic once	0.07 (-0.03, 0.18)	0.174
	Recurrent parasitaemia	0.69 (0.56, 0.83)	**<0.001**
	Sickle Trait	-0.11 (-0.24, 0.02)	0.093
	alpha –Thal. Heterozygous	0.06 (-0.04, 0.16)	0.24
	alpha –Thal. Homozygous	-0.01 (-0.13, 0.12)	0.937
	Prior Parasitaemia	0.08 (0.06, 0.1)	**<0.001**

Notes

Multivariable generalized estimating equation (GEE) with exchangeable correlation structure, adjusted for changes in malaria transmission (n=287).

[‡] GEE regression coefficients indicating reduction/increase in the antibody response.

[Δ] *P*-values ≤ 0.05 indicated in bold type.

4.3.6 Antibody responses to merozoite antigens are better maintained by older children in the face of declining malaria transmission

With an overall decline in antibody responses over the study period, I decided to investigate the effect of the various factors described above on the maintenance/decay of IgG responses in the later parts of the study when malaria transmission had fallen considerably. Parasite prevalence during the May 2004 cross sectional bleed was 6.0% and dropped to 3.7% in October 2004. In the months between sampling 98% of the children did not exhibit any detectable parasitaemia during the weekly surveillance visits. GEE models were used to quantify the decline in antibody levels in only those children who where sero-positive for the antigen tested in May 2004 and did not have any detectable parasitaemia in the subsequent interval. In general, the decline in malaria transmission resulted in a reduction of antibody levels of around 0.2 OD units for anti-AMA1 responses and 0.3 OD units for anti-MSP2 responses (Tables 4.12 and 4.13; Appendix 2, Tables 1-3). Univariate analysis indicated that the youngest children (0-2 years) had the greatest reduction in antibody levels to both AMA1(W2mef) (0.50 OD units, p=0.005) (Table 12) and MSP2(3D7) (0.91 OD units, p<0.001) (Table 4.13). This was also the case for the other AMA1 and MSP2

variants tested (Appendix 2, Tables 1-3). Conversely, the oldest children (6-9 years) better maintained their antibody levels to both AMA1 and MSP2 ($p<0.05$) (Table 4.12 and 4.13; Appendix 2, Tables 1-3). It was only the 6-9 year olds who maintained their antibody responses to AMA1 variants at significantly higher levels compared to 0-2 year-olds ($p<0.05$) (Table 4.12; Appendix 2, Tables 1 and 2). This was not the case for the antibody responses to MSP2 variants (Table 4.13; Appendix 2, Table 3).

Table 4.12 Differences in the rate of anti-AMA1(W2mef) IgG decay between May and October 2004

	Anti-AMA1(W2mef) IgG		
	β (95% CI)[ǂ]	*P*-value[Δ]	N[ε]
Sample time[1]	-0.21 (-0.27, -0.15)	**<0.001**	86
Age 0-2 years	-0.5 (-0.85, -0.15)	**0.005**	8
Age 3-5 years	-0.27 (-0.37, -0.17)	**<0.001**	24
Age 6-9 years	-0.15 (-0.22, -0.08)	**<0.001**	54
Compared to 0-2 year olds			
... Age 3-5 years	0.33 (-0.18, 0.84)	0.209	86
... Age 6-9 years	0.56 (0.08, 1.03)	**0.021**	
HbAA (Normal)	-0.22 (-0.29, -0.15)	**<0.001**	74
HbAS (Sickle trait)	-0.15 (-0.32, 0.02)	0.088	9
Compared to HbAA children			
..... HbAS	-0.19 (-0.64, 0.27)	0.421	83
Alpha-thalassemia NORM[2]	-0.23 (-0.32, -0.15)	**<0.001**	31
Alpha-thalassemia HET[2]	-0.21 (-0.33, -0.09)	**<0.001**	41
Alpha-thalassemia HOM[2]	-0.11 (-0.15, -0.07)	**<0.001**	10
Compared to normal children			
.... Alpha-thalassemia HET	-0.02 (-0.33, 0.28)	0.876	82
.... Alpha-thalassemia HOM	-0.35 (-0.81, 0.11)	0.136	

Notes

Univariate generalized estimating equation (GEE) with exchangeable correlation structure.

[ǂ]GEE regression coefficients indicating reduction/increase in the rate of antibody decay between May and October 2004.

[Δ]*P*-values ≤ 0.05 indicated in bold type.

[ε]Number of samples included in the model.

[1]Change in IgG response between May and October 2004.

[2]Alpha-thalassemia genotypes: NORM = normal, HET = heterozygous, HOM = homozygous.

Table 4.13 Differences in the rate of anti-MSP2(3D7) IgG decay between May and October 2004

	Anti-MSP2(3D7) IgG		
	β (95% CI)[‡]	*P*-value[Δ]	N[£]
Sample time[1]	-0.34 (-0.52, -0.15)	**<0.001**	32
Age 0-2 years	-0.91 (-1.41, -0.41)	**<0.001**	3
Age 3-5 years	-0.56 (-1.07, -0.06)	**0.030**	7
Age 6-9 years	-0.19 (-0.35, -0.02)	**0.032**	22
Compared to 0-2 year olds			
... Age 3-5 years	-0.34 (-1.15, 0.47)	0.412	32
... Age 6-9 years	0.06 (-0.66, 0.79)	0.863	
HbAA (Normal)	-0.41 (-0.58, -0.25)	**<0.001**	27
HbAS (Sickle trait)	0.08 (-0.61, 0.76)	0.830	5
Compared to HbAA children			
..... HbAS	-0.32 (-0.9, 0.27)	0.286	32
Alpha-thalassemia NORM[2]	-0.31 (-0.57, -0.05)	**0.021**	14
Alpha-thalassemia HET[2]	-0.29 (-0.58, -0.01)	**0.043**	14
Alpha-thalassemia HOM[2]	-0.27 (-0.57, 0.03)	0.083	3
Compared to normal children			
.... Alpha-thalassemia HET	-0.06 (-0.49, 0.38)	0.804	31
.... Alpha-thalassemia HOM	-0.76 (-1.5, -0.02)	**0.043**	

Notes

Univariate generalized estimating equation (GEE) with exchangeable correlation structure.

[‡]GEE regression coefficients indicating reduction/increase in the rate of antibody decay between May and October 2004.

[Δ]*P*-values ≤ 0.05 indicated in bold type.

[£]Number of samples included in the model.

[1]Change in IgG response between May and October 2004.

[2]Alpha-thalassemia genotypes: NORM = normal, HET = heterozygous, HOM = homozygous.

There was no difference in the decay rate of antibody responses to AMA1 and MSP2 in children with the sickle trait compared to those without the trait (Tables 4.12 and 4.13; Appendix 2, Tables 1-3). When analysed separately, children who were homozygous for alpha-thalassemia appeared to maintain their antibody response to AMA1 better (reduction of 0.08-0.11 OD units, $p<0.05$) than normal (reduction of 0.17-0.23 OD units, $p<0.001$) or heterozygous children (reduction of 0.21-0.23 OD units, $p<0.001$) (Table 4.12; Appendix 2, Tables 1 and 2). When this difference seen in homozygous children was further analysed, it was not significantly different to the decay rates of antibody responses to AMA1 seen in normal children. Interestingly, children homozygous for alpha-thalassemia had significant declines in their antibody response to MSP2(3D7) ($p=0.043$) (Table 4.13) but not to MSP2(FC27) (Appendix 2, Table 3) when compared to normal children.

4.4 Discussion

Longitudinal analysis of antibody responses to AMA1 and MSP2 revealed three overall patterns: (i) consistently high antibody responses, (ii) fluctuating antibody responses, and (ii) consistently low antibody responses. A large proportion of the study participants had consistently low antibody responses to both AMA1 and MSP2. Children with consistently low IgG responses may have not been exposed at sufficient levels to *Plasmodium* over the study period and therefore did not have a measurable antibody response to the antigens tested. These children could also include a subgroup of non- or poor-responders as a result of host genetic or other factors. An alternative explanation could be that the sampling interval of six months could result in a loss of detection of significant antibody responses due to short IgG half-life, as suggested in some studies. More children had persistent antibody responses to AMA1 above 2 OD units compared to the MSP2 antibody response. In the analysis of cross-sectional data (chapter 3), an overall lower antibody response was seen with regards to MSP2 compared to AMA1. With these results in mind, it is not surprising to observe a lower antibody response over the study period. As these are the same children, this result may be indicating a relative lower immunogenic capacity of MSP2 compared to that of AMA1. AMA1 is a structurally constrained antigen whereas MSP2 is not so these results may indicate that the conformations adopted by the recombinant MSP2 antigen on the ELISA plate could be less common than the conformations presented to the immune system in natural infection, which would lead to less recognition of this antigen in the assay. As IgG responses to MSP2 were tested at serum dilutions of 1:1000 as opposed to those to AMA1 variants which were tested at 1:4000 dilutions, I am reasonably confident that the responses to MSP2 were genuinely of a lower magnitude than those to AMA1. Both antigens were of high purity (J. Beeson and R. Anders, personal communication) and were used to coat ELISA plates at a saturating concentration. The higher IgG titres to MSP2(3D7) is likely to reflect a greater incidence of infections with isolates having the MSP(3D7) allelic type compared to FC27. This is in agreement with a study carried out in the same area that found type A *msp2* alleles in 86.9% of asymptomatic infections in children below the age of 11 years compared with 41.3% for type B *msp2* alleles (Farnert et al. 2009).

Antibody responses to merozoite antigens in endemic populations have been measured in other studies and are generally reported to be short-lived in children (Fruh et al. 1991; Branch et al. 1998; Cavanagh et al. 1998; Soares et al. 1999; Udhayakumar et al. 2001; Kinyanjui et al. 2007; Akpogheneta et al. 2008; Biswas et al. 2008). Though the exact half life of anti-merozoite antibodies is unknown, studies indicate a range of a week to 4 months (Kinyanjui et al. 2007; Akpogheneta et al. 2008). The sampling framework in this study was approximately every 5-6 months which inevitably made it impossible to pick up fine trends in IgG boosting as suggested by other studies (Branch et al. 1998; Cavanagh et al. 1998; Kinyanjui et al. 2007; Akpogheneta et al. 2008). However, findings from this cohort suggest that at least a proportion of children sustained antibodies for several months after malaria transmission reduced substantially.

Antibody 'response rankings' within this study population were steadier with regards to AMA1 compared to MSP2 as observed in the tracking plots (Figure 4.2). Most children appeared to maintain their response (whether high or low) relative to others in the same age group though a small number did have widely varying responses. Assuming that the population of circulating parasites over the study period displayed all the antigens tested in comparable frequencies with comparable exposure, one could infer that the increased prevalence of stable responses above the cohort mean to AMA1 could be indicative of higher AMA1 immunogenicity and better maintenance of the anti-AMA1 response. Children were as equally able to mount an immune response to AMA1 as to MSP2 that was dependent on their overall 'responsiveness' and levels of exposure. Though AMA1was more immunogenic than MSP2 (eliciting higher IgG titres), the study population was not predisposed to responding more to one merozoite antigen than the other. That anti-AMA1 IgG responses saturate faster in endemic populations and appear to be maintained better than those to MSP2 has been noted in other studies (Udhayakumar et al. 2001; Drakeley et al. 2005; Morais et al. 2006; Akpogheneta et al. 2008). When the responses to both AMA1 and MSP2 were investigated in selected children, the responses to AMA1 and MSP2 tended to be similar across variants in older children. Though not implicit, this could imply a trend towards antibody responses to conserved epitopes on both AMA1 and MSP2 as children get older, or that greater exposure leads to the larger repertoire of antibodies to different antigens and their variants. Some data suggests that a substantial component of antibodies to recombinant AMA1 measured by

ELISA is against conserved epitopes, whereas antibodies to MSP2 show a higher degree of allele-specific reactivity (Stanisic et al. 2009). This has also been suggested by unpublished data by Beeson J *et al.* Due to time constraints, it was not possible to address this issue in this thesis using competition ELISAs with different alleles or other approaches.

At a population level, mean antibody levels to all five antigens tested declined over the study period. These responses paralleled the decline in malaria transmission seen over the same period. Antibody responses to AMA1(W2mef) increased from May 2002 before declining and reaching levels comparable to those of the other two AMA1 variants in October 2003. Though no data on circulating parasites is available for this period, this antibody response may indicate an increase in the frequency of parasites with this AMA1 variant. There was an increase in the incidence of both recorded parasitaemia and clinical disease in this area between May 2002 and May 2003 which could possibly be due to new circulating parasite isolates that may have been carrying an AMA1 variant similar to, if not the W2mef type. Whilst parasite frequencies cannot be determined simply by examining antibody data, this data may give some insight into the circulating parasite strains as seen in other longitudinal studies (Branch et al. 1998; Cavanagh et al. 1998; Giha et al. 1999). Presently there is little data published on the sero-epidemiology of different alleles of AMA1, particularly with respect to understanding population exposure to different alleles. This knowledge will be important for advancing vaccine development of AMA1.

Individual plots of antibody levels showed more stability in the responses to all three AMA1 variants as children aged compared to MSP2, although fluctuating antibody responses were still observed in older children. These results were in agreement with two studies which showed persistent and stable antibody responses to AMA1 and MSP2 (Riley et al. 1993; Udhayakumar et al. 2001). Though these prior studies were both carried out in adults, the results described here show that a stable antibody response to AMA1 can be detected over time from as early as 5 years of age. The difference observed with MSP2 compared to AMA1 could be due to both its relative immunogenicity and the nature of the recombinant protein, as mentioned earlier. The mean smoothed antibody patterns showed that older children always had higher antibody responses to AMA1 and MSP2 over time. This occurred without any noticeable increase over time in the mean antibody responses exhibited by the younger children. All children showed a decline in antibody

responses over the study period that implied a strong effect of malaria transmission, particularly for the last year of the study. As cross-sectional studies show an increase in antibody responses with age, we may expect some sort of positive trajectory in longitudinal antibody levels over time as children get older. Although antibody responses to AMA1 and MSP2 were indeed higher in older children than younger ones, there was no increase in the antibody response of younger children with time. This could indicate a lack of cumulative exposure as malaria transmission declined over the study period and separated the effect of age from cumulative exposure which has not been possible to quantify in cross-sectional studies. Only one study that measured anti-MSP1 responses in infants over their first year of life has also shown no change in the magnitude of antibody responses with age (Branch et al. 1998). Children with the sickle trait as well as those homozygous for alpha-thalassemia had consistently lower antibody responses than their normal counterparts throughout the study period. This could also be an effect of the overall reduced exposure to *Plasmodium* that was discussed in chapter 3.

Cross-sectional studies all show that concurrent parasitaemia at sampling time is associated with higher antibody levels (Al-Yaman et al. 1995; Bull et al. 2002; Metzger et al. 2003; Kinyanjui et al. 2004; Polley et al. 2006). This was also the case when analysed longitudinally. Children who had concurrent parasitaemia more than once at sample time had higher antibody responses throughout the study period compared to those with concurrent parasitaemia only once or never. These results were further corroborated when investigation into recorded parasitaemia in the intervals was carried out. Children who had recorded parasitaemia in the intervals and also had concurrent parasitaemia once at the cross sectional bleeds were also the children with very high antibody responses to both AMA1 and MSP2. The levels were only higher in those children who were parasitaemic at more than one sample point though the difference was not statistically significant. In the case of all antigens, higher exposure led to a higher antibody response. A limitation of the study is that we may have missed some instances of exposure. During the weekly surveillance, blood smears were only carried out when children had fever or a history of fever or other illness so any asymptomatic parasitaemia – other than that caught at sample time – cannot be identified in this analysis. Additionally, parasitaemia was only defined as detected by microscopy, which is known to miss low level parasitaemias that are detectable by PCR (Okell et al. 2009). One could

argue that this is not a problem as only those instances that have led to a child mounting a significant immune response (fever being an indication of an active immune response) means that the particular incident of exposure is important and is probably the type that would result in measurable antibody responses, though we cannot rule out the contribution of sub-patent parasitaemia to antibody induction. On the other hand asymptomatic parasitaemia could be reflective of effective humoral and cellular immune function. In this particular case, significant antibody responses were only detected when parasitaemia was actually present (at sample time as well as the intervals) which could indicate that longitudinal antibody responses give an overall indication of levels of exposure. The finding that the factor with the greatest effect on antibody levels to AMA1 and MSP2 was concurrent exposure, as well as the fact that children with concurrent parasitaemia were 1.5 times more likely to be parasitaemic in the intervals adds to the evidence that antibody levels are a marker of exposure. These results are comparable to those seen in a study which found that those children who were parasitaemic at baseline went on to experience more instances of parasitaemia over the study period (Polley et al. 2004; Akpogheneta et al. 2008). The finding that children with concurrent parasitaemia were also more likely to have parasitaemic episodes in the intervals is in agreement with published data on this population which showed that 20% of the children had (those parasitaemic at cross-sectional bleeds) experienced 56% of all malaria episodes (Mwangi et al. 2008).

With the apparent decline in malaria globally, it is important to understand how this affects the acquisition and maintenance of antibodies, and there is considerable interest in this question in the field. The extended duration of this cohort and repeated sampling provided important early insights into the potential effects of declining malaria on the acquisition and maintenance of responses that are believed to play a role in immunity. As the incidence of malaria and prevalence of *P. falciparum* infections declined in the latter part of the study, there was a reduction in the levels and prevalence of antibodies to all three AMA1 variants and the two MSP2 alleles. By October 2004, the prevalence of antibodies to AMA1 had declined to 36.1% (W2mef), 35.8% (HB3), 34.1% (3D7) and those to MSP2 to 8.9% (3D7) and 8.6% (FC27) , compared to 45.5%, 48.1%, 43.2%, 28.6%, and 23.1% respectively in May 2002. The difference in the prevalence and levels of antibodies at the start compared to the end of the study was particularly evident among young

children. Although there was a lengthy period between cross sectional bleeds, it is possible to make broad comparisons and quantification in the rate of decay of IgG responses to AMA1 and MSP2. The results here showed that anti-MSP2 antibodies declined at a higher rate than those to AMA1 which is in agreement with a recent study carried out in The Gambia on children less than 6 years of age (Akpogheneta et al. 2008). In agreement with the study mentioned, the rate of anti-AMA1 IgG decay was lower in older children but this was not the case for anti-MSP2 antibodies. These results point both to differential effects of age on antibody decay and antigen-specific effects. The fact that antibody responses to AMA1 and MSP2 were maintained at significantly higher levels in older children throughout the study duration gives further support to an independent effect of age. Older children may have a larger population of long-lived plasma B cells which is in agreement with the fact that both T-helper cell function and antigen presentation increases with age in children (Clerici et al. 1993). Akpogheneta *et al* suggested that the tandem repeated epitope sequences in MSP2 (which AMA1 does not contain) could reduce the effectiveness of MSP2 to induce B cell memory responses.

A number of limitations of this study are (i) the long periods between cross sectional bleeds, (ii) the lack of isolate typing, and (iii) the possibility that a number of episodes of *P. falciparum* exposure could have been missed in the intervals due to the inclusion criteria for blood smears. Only those children with fever or with a history of fever in the last week had blood smears made. This means that we may have missed a considerable number of asymptomatic episodes over the 2. 5 years of this study. A major strength of this study was the dynamic shift of malaria transmission in the study area. Without the effects of continuous infection (or superinfection) which is found in areas of high transmission, it was possible to investigate the acquisition of anti-merozoite antibodies in a situation that may be very relevant to the emerging malaria epidemiology in Africa today. Falling malaria transmission rates along with increased use of bednets across the continent has led a significant decline in the incidence of malaria (Bhattarai et al. 2007; Fegan et al. 2007; Okiro et al. 2007). Though the predicted effect of this decline on acquisition to malaria is a corresponding decline in the immunity to malaria in endemic populations, it has yet to be presented in detail. To my knowledge, this study is the first to do so during the time when children are actively acquiring immunity to *Plasmodium*.

Antibody levels are dynamic outcomes that are dependent on a number of changing factors. Cross-sectional studies attempt to assign a static category of responsiveness according to antibody levels measured at baseline. This study has used established statistical methods to quantify the amount of change occurring over time which is a truer representation of an individual's immune response to malaria. An important question that this study addresses is whether antibody responses measured at a single time point sufficiently capture enough information to assess an individual's antibody responsiveness or immune status compared to longitudinal measurement. Antibody responses to merozoite antigens (MSP1 and MSP2) at a single time point have been suggested to reflect age-related cumulative exposure over an extended period, although it was acknowledged that this may not be the case for highly immunogenic antigens such as AMA1 (Drakeley et al. 2005). Indeed, the present study suggests that antibody responses to AMA1 in children may be highly indicative of recent exposure and parallel ongoing changes in malaria transmission. The observation that mean anti-AMA1(W2mef) antibodies increased in the population when there was a higher incidence of malaria, and then declined with decreasing transmission, supports this theory.

Studies of protection from clinical disease in association with antibody levels give conflicting results. For some, antibodies are associated with protection, whereas for others it is not the case. These results may be due to the fact that the groupings used – high or low responders, tertiles, parasitaemic or aparasitaemic – are each made up of individuals with varying amounts of exposure and responsiveness. The antibody levels at a single time point are affected by not only age and concurrent parasitaemia, but also by the prior exposure and their prior antibody responses. Low antibody levels could be due to a lack of exposure or an appropriate immune response (humoral and cellular) sometime in the past which has led to control of parasitaemia to levels that don't activate a high antibody response. This study found that children with more exposure maintained high antibody responses to merozoite antigens which fell as malaria transmission declined. Increasing age and parasitaemia at a sampling were also associated with higher antibody responses. Malaria transmission appeared to have a great effect on the antibody response as the younger children in the cohort failed to mount increasing antibody responses as they aged. The association of these antibody responses with protection against malaria will be assessed in the following chapter.

5 The association of anti-merozoite antibody responses with protection from clinical malaria

5.1 Introduction

The inferred role of antibodies in protection against malaria in endemic settings has traditionally been elucidated by the measurement of antibodies in serum at a single time point followed by surveillance of study subjects over a period of time for incidence of malaria disease. These association studies have been carried out in various settings examining antibody responses to several different *Plasmodium* – predominantly merozoite – antigens. Published results often conflict in their assessment of the protective capacity of antibodies to merozoite antigens in protecting against clinical malaria. To date, most of the published data on the association of anti-merozoite antibodies with protection against malaria is on MSP1. Antibodies to the C-terminal ($MSP1_{19}$ and $MSP1_{42}$) and N-terminal regions (K1-like, MAD20-like, and RO33-like) have been associated with protection against malaria in numerous studies (Riley et al. 1992; Hogh et al. 1995; Al-Yaman et al. 1996; Egan et al. 1996; Branch et al. 1998; Conway et al. 2000; Polley et al. 2003; Cavanagh et al. 2004; Perraut et al. 2005; Dodoo et al. 2008; Osier et al. 2008; Stanisic et al. 2009). This is not always the case; other studies have associated the same antibody responses with an increased risk of malaria (Dodoo et al. 1999; Conway et al. 2000; Polley et al. 2003; Cavanagh et al. 2004; John et al. 2005; Gray et al. 2007; Nebie et al. 2008; Osier et al. 2008). A recent systematic review and meta-analysis of prospective cohort studies that investigated the protective effects of antibody responses to merozoite antigens (AMA1, EBA175, GLURP, MSP1, MSP2, and MSP3) showed that antibody responses to $MSP1_{19}$ and the C-terminus of MSP3 reduced the risk of *P. falciparum* malaria by 18% and 54% respectively, and antibodies to AMA1 were associated with protection in most studies (Fowkes et al. 2010). In this meta-analysis, there was no significant overall association of antibody responses to MSP2, full length MSP3, or N-terminal regions of MSP1 with protection from malaria.

Two studies carried out in the same part of Kenya provide evidence that antibodies to AMA1 are associated with protection. Anti-AMA1 antibodies to the 3D7 and FVO variants were associated with protection from clinical malaria in children and adults over 6 months of follow-up (Polley et al. 2004). A later study on samples from the same area on children showed that high levels of antibody reactivity was associated with a reduced risk of hospital admission over 8 months of follow-up (Osier et al. 2008). Although antibodies to AMA1 were predictive of protection in this study; it was only when anti-AMA1 antibodies were analysed in combination with those to MSP2 or MSP3 that the protection was significant. A similar result had also been obtained from a study carried out in The Gambia where antibody reactivity to both AMA1 and MSP2 in combination was more predictive of protection (Gray et al. 2007). In a study with 4 months of follow-up that was carried out on children aged 6 months to 15 years, total IgG responses to AMA1(FVO) were not associated with protection against malaria yet IgG1 responses to the same antigen were associated with protection (Nebie et al. 2008). Though high levels of IgG to AMA1 were also associated with protection in a study performed in Papua New Guinea, the association with protection was significant for IgG3, but not IgG1 (Stanisic et al. 2009). Subclass responses to merozoite antigens will be discussed in detail in the following chapter.

The earliest evidence of anti-MSP2 antibodies being associated with protection against malaria came from studies in Papua New Guinea. High antibody levels to MSP2 were associated both with fewer episodes of fever and less anaemia (Al-Yaman et al. 1994). A later study in the same place showed that anti-MSP2 (3D7) antibodies were associated with a reduced risk of clinical malaria in the subsequent year (Al-Yaman et al. 1995). In Kenya, high antibody levels to MSP2 were associated with protection from malaria over 28 weeks (Polley et al. 2006). Interestingly, a single study showed an association with protection when high anti-MSP2 IgG3 antibodies were present and an increased risk of malaria with high anti-MSP2 IgG1 levels (Taylor et al. 1998) whereas another study showed protection from clinical disease for both IgG1 and IgG3 responses to MSP2 (Metzger WG et al, 2003). More evidence for the protective effect of anti-MSP2 total IgG and IgG3 was seen in a study carried out in Senegal (Sarr et al. 2006). Another recent study has also shown that anti-MSP2 antibodies were associated with a reduced risk of malaria but are not associated with protection from re-infection *per se* (Stanisic et al. 2009).

This chapter aims to investigate the association between antibody responses to merozoite antigens and protection against malaria. Two representative merozoite antigens were selected for investigation here; AMA1, which is located in the apical organelles and plays an important role in invasion, and MSP2, which is an abundant merozoite surface antigen. Additionally, both antigens are leading vaccine candidates. The malaria literature is generally in agreement that antibodies contribute a large part of the natural defence against disease. A major issue has been the lack of agreement on the protective effects of different antibody responses to malarial antigens. Contradictory results between studies could be attributed to several factors including cohort and antigen differences, malaria transmission, follow-up time, and analysis methods. Very few studies have examined antibody responses over an extended period of time (such as over several seasons) and analysed associations between antibodies and protective immunity using data from multiple sampling points. Due to the extended longitudinal nature of this study, along with the changes in malaria transmission described in previous chapters, this study provides a unique opportunity for investigating the role of antibody responses in protection from malaria. As immunity to malaria develops over time and is dependent on the malaria transmission intensity (Marsh and Kinyanjui 2006) this study will attempt to assess the acquisition of immunity under different malaria transmission intensities. This will be done by first identifying factors other than antibody responses that affect the risk of malaria and then two different methods will be used to analyse whether antibody responses to AMA1 and MSP2 may be protective in this cohort.

5.2 Methods

5.2.1 Statistical Analysis

Two different analytical approaches were used to investigate the association between antibody responses and protection from clinical malaria. The first utilised the data in a cross sectional cohort format and analysed antibody responses at baseline with the risk of malaria on follow-up. The second method investigated the association between antibody responses throughout the study with the risk of malaria during the study. The results from these two analysis methodologies were then compared. Significance was reached at the 0.05 level for all tests. *P* values ranging from 0.06 to 0.1 were considered as borderline significance, providing weaker evidence for the association being examined.

5.2.1.1 Risk analysis with cross sectional data format

In order to investigate whether the association between antibodies and risk of disease was dependent on the time that sampling occurred, univariate and multivariate generalised linear models (GLM) were used with data collected and antibody measurements at the May and October 2002 bleed times as well as those taken during the May 2003 bleeds. From October 2003 there were no recorded episodes of clinical malaria in this cohort so antibody measures for samples collected after the May 2003 bleed were not included in the analysis. Univariate analysis was first carried out to assess the non-antibody factors that affected the risk of clinical disease. These factors were age, concurrent parasitaemia, and haemoglobinopathies. Each was included in the model in different ways. Age was a continuous variable, concurrent parasitaemia was coded as both a binary variable (at sample time) and also as a categorical variable when it described an individual's ongoing parasitaemic status over the study period (never parasitaemic, parasitaemic once, and recurrent parasitaemia). Sickle trait was a binary variable and alpha-thalassemia a categorical variable (normal, heterozygous, homozygous). As mentioned earlier, univariate analysis was carried out on the May and October 2002 samples as well as the May 2003 samples. It was then carried out on all the samples in the study using the same model but including a clustering indicator that identified repeated sampling on the same individuals but ignored the longitudinal nature of the sampling. Each antibody response was inputted into the model as a

continuous variable. Multivariable GLM analysis for the risk of a clinical episode was carried out on the antibody responses for the May 2002 to May 2003 bleeds as well as for all samples and was adjusted for age, concurrent parasitaemia, and haemoglobinopathies.

5.2.1.2 Risk analysis with longitudinal data format

The overall risk of a clinical episode of malaria in this study was assessed using Generalised Estimating Equations (GEE) for 'count' outcome variables. GEE allow for the analysis of longitudinal relationships using all available longitudinal data without summarizing the longitudinal outcome into a single value. The GEE for 'count' outcomes is comparable to a poisson regression analysis except that within-subject correlation is taken into account. Exchangeable correlation structure which assumes that correlations between subsequent measurements are assumed to be the same irrespective of the length of the time interval was used in this case. Univariate GEE analysis on antibody responses and the non-antibody factors that may affect the risk of clinical malaria was carried out followed by multivariable analysis on the adjusted effect of antibody responses to AMA1 and MSP2. Recently published results from a separate study carried out in Kilifi has shown that removal of those individuals without any exposure strengthened the protective effects of some malarial antigens against clinical disease (Bejon et al. 2009). With this in mind, I carried out the multivariable analysis on only those children who had recorded exposure to *Plasmodium* over the study period (as indicated by a positive blood smear in during the weekly surveillance or at sample time). All the antibody data obtained was included in the GEE model and was inputted as a continuous variable.

5.3 Results

5.3.1 Factors affecting the risk of malaria six months post-sampling

In order to investigate the protective effects of antibody responses to AMA1 and MSP2, this study was divided into 6-monthly intervals based on the timing of cross-sectional bleeds. Protection was assessed from each sample time for a period of six months until the next sampling, which is similar to the approach that has typically been used in the literature (Fowkes et al. 2010). Each sample time was considered baseline with no regard being taken of antibody measures or exposure before

each sampling frame. From October 2003 no children in this cohort experienced any clinical malaria due to dropping malaria transmission in the community. As the outcome was absent from October 2003, only the first three sampling times were used in this analysis. In the six months post sampling in May 2002 there were 29 episodes of malaria. Following the October 2002 cross sectional bleed there were 156 episodes of malaria. This number fell to 78 episodes after the May 2003 cross sectional bleed.

Factors other than antibody responses that could affect the risk of clinical malaria were first investigated using univariate models. For all three sampling times key demographic characteristics remained the same. Females made up 44.5%, 44.4%, and 44.5% of the cohort respectively. The mean and median ages at each sampling was; 3.8/3.7 years, 4.0/3.9 years, and 4.4/4.3 years respectively. The only difference at each sampling time was the levels of malaria transmission (inferred by the proportion parasitaemic at sampling); 51%, 21%, and 38%.

When examined at the three sampling times mentioned, key factors that are known to influence the risk of clinical malaria had similar effects. Age had little or no effect on the risk of malaria in May 2002 (IRR 1.11, 95% CI 0.93-1.32, p = 0.26), October 2002 (IRR 1.07, 95% CI 1.00-1.15, p = 0.05), or May 2003 (IRR 1.02, 95% CI 0.93-1.12, p = 0.63) (Table 5.1). Concurrent parasitaemia at time of sampling at May 2002 (IRR 1.58, 95% CI 0.68-3.71, p = 0.29), October 2002 (IRR 1.11, 95% CI 0.63-1.96, p = 0.71) and May 2003 (IRR 0.99, 95% CI 0.51-1.92, p = 0.98) had no significant effect on the risk of malaria in the subsequent six months. At all three sampling times the presence of the sickle trait was associated with a reduced risk of clinical malaria, however, this was significant only in October 2002 - May 2002 (IRR 0.27, 95% CI 0.04-1.98, p = 0.20), October 2002 (IRR 0.49, 95% CI 0.26-0.92, p = 0.03), and May 2003 (IRR 0.52, 95% CI 0.23-1.20, p = 0.13). Interestingly, being alpha-thalassemia homozygous was associated with a decreased risk of malaria subsequent to the May 2002 (IRR 0.39, 95% CI 0.11-1.37, p = 0.14) and the May 2003 (IRR 0.52, 95% CI 0.26-1.06, p = 0.07) cross sectional bleeds though this association was only of borderline significance in May 2003. The association between both these haemoglobinopathies and a reduced risk of malaria in this cohort has been previously reported (Williams et al. 2005a; Williams et al. 2005b; Williams et al. 2005c).

Table 5.1 Factors affecting the risk of clinical malaria in the 6 months subsequent to sampling time

	Malaria episodes[‡]	Univariate analysis[◊]	IRR (95% CI)	*P*-value
All samples[Δ]	273 (132)	Age (years)	0.99 (0.93, 1.04)	0.61
		Parasitaemia[1]	1.12 (0.71, 1.76)	0.62
		- Parasitaemic once[‡]	2.05 (1.49, 2.83)	**<0.001**
		- Recurrent parasitaemia[‡]	1.00 (0.60, 1.68)	0.99
		Sickle trait	0.45 (0.24, 0.84)	**0.01**
		Alpha-thal HET[2]	0.91 (0.64, 1.32)	0.63
		Alpha-thal HOM[2]	0.74 (0.46, 1.19)	0.22
May 2002[†]	29 (22)	Age (years)	1.11 (0.93, 1.32)	0.26
		Parasitaemia[1]	1.58 (0.68, 3.71)	0.29
		Sickle trait	0.27 (0.04, 1.98)	0.20
		Alpha-thal HET[2]	0.65 (0.30, 1.4)	0.27
		Alpha-thal HOM[2]	0.39 (0.11, 1.37)	0.14
October 2002[†]	156 (99)	Age (years)	1.07 (1.00, 1.15)	**0.05**
		Parasitaemia[1]	1.11 (0.63, 1.96)	0.71
		Sickle trait	0.49 (0.26, 0.92)	**0.03**
		Alpha-thal HET[2]	1.04 (0.74, 1.47)	0.83
		Alpha-thal HOM[2]	0.96 (0.6, 1.51)	0.85
May 2003[†]	78 (61)	Age (years)	1.02 (0.93, 1.12)	0.63
		Parasitaemia[1]	0.99 (0.51, 1.92)	0.98
		Sickle trait	0.52 (0.23, 1.20)	0.13
		Alpha-thal HET[2]	0.76 (0.48, 1.21)	0.25
		Alpha-thal HOM[2]	0.52 (0.26, 1.06)	**0.07**

Notes

[‡] Total number of malaria episodes recorded, number of children experiencing malaria indicated in brackets.

Malaria definition: Temperature ≥37.5˚C with patent parasitaemia in <1 year-olds or parasitaemia ≥2500 parsites/µl in children >1 year of age.

[◊] Univariate poisson regression.

[Δ] Analysis of all samples (all 6 sampling points) with the addition of an indicator for those children contributing more than one observation to the analysis.

[†] Analysis covering the 6 month period subsequent to the cross sectional bleed indicated.

No analysis for October 2003, May 2004, and October 2004 cross-section bleeds due to lack of malaria episodes after the October 2003 sampling.

[1] Concurrent parasitaemia at sampling.

[‡] Number of times with concurrent parasitaemia at sampling over entire study period; once and recurrently (more than once).

[2] Alpha-thalasssemia: HET = heterozygous, HOM = homozygous.

P-values ≤0.1 indicated in bold.

A second analysis using all the samples in the study (May 2002 to October 2004) with the inclusion of an identifier that indicated individuals had been sampled repeatedly was carried out. As a number of the children had concurrent parasitaemia at more than one of the cross sectional bleeds, it was possible to group the children according to this. This grouping of parasitaemia was an indicator of each child's level of exposure. The children were categorised as never parasitaemic, parasitaemic once, and recurrently parasitaemic at cross sectional sampling. This analysis showed that presence of concurrent parasitaemia at least once over the study period was associated with a significantly increased risk of clinical malaria (IRR 2.05, 95% CI 1.49-2.83, $p < 0.001$) whereas the

presence of the sickle trait was associated with a reduced risk of clinical malaria (IRR 0.45, 95% CI 0.24-0.84, $p = 0.01$) (Table 5.1).

5.3.2 The association of antibody responses with protection from malaria six months post-sampling is dependent on malaria transmission at sampling

Univariate analysis of the effect of antibodies to AMA1 and MSP2 on the risk of clinical malaria over six months post-sampling was carried out on the May 2002, October 2002, and May 2003 samples. As indicated in previous chapters, a major determinant of the antibody responses to AMA1 and MSP2 in this cohort was the intensity of malaria transmission. Therefore, any differences in the association of antibody responses to the same antigen with the risk of clinical malaria at different time points could be attributed to changes in malaria transmission at sampling (Table 5.2).

When each cross sectional bleed was analysed separately, only at the May 2002 bleed were there any antibody responses that were weakly associated with the risk of malaria. The strongest association was that of antibody responses to MSP2(FC27). These antibodies were associated with an increased risk of malaria though this was of borderline significance (IRR 1.52, 95% CI 0.97-2.40, $p = 0.07$). Responses to MSP2(3D7) appeared to reduce the risk of malaria (IRR 0.54, 95% CI 0.26-1.15, $p = 0.11$) whereas those to AMA1(HB3) appeared to increase the risk (IRR 1.36, 95% CI 0.88-2.12, $p = 0.17$), though both of these associations were not significant. Though not significant, antibodies to all three AMA1 variants tested appeared to be associated with an increased risk of malaria in the six months following the May 2002 bleed (Table 5.2). This was not the case for MSP2; antibodies to one variant appeared to be associated with protection – MSP2(3D7) – whereas antibodies to MSP2(FC27) appeared to be associated with an increased risk of malaria.

Table 5.2 The associations between total IgG to AMA1and MSP2 and the risk of clinical malaria in the 6 months subsequent to sampling time

	Univariate analysis[◊]	IRR (95% CI)	*P*-value
All samples[Δ]	AMA1(W2mef)	1.46 (1.23, 1.72)	**<0.001**
	AMA1(HB3)	1.11 (0.89, 1.38)	0.36
	AMA1(3D7)	1.09 (0.87, 1.38)	0.46
	MSP2(3D7)	0.77 (0.59, 1.01)	**0.06**
	MSP2(FC27)	1.20 (0.94, 1.55)	0.15
May 2002[†]	AMA1(W2mef)	1.33 (0.86, 2.06)	0.21
	AMA1(HB3)	1.36 (0.88, 2.12)	0.17
	AMA1(3D7)	1.36 (0.85, 2.16)	0.20
	MSP2(3D7)	0.54 (0.26, 1.15)	0.11
	MSP2(FC27)	1.52 (0.97, 2.40)	**0.07**
October 2002[†]	AMA1(W2mef)	1.08 (0.9, 1.28)	0.42
	AMA1(HB3)	1.04 (0.8, 1.35)	0.76
	AMA1(3D7)	1.03 (0.79, 1.36)	0.81
	MSP2(3D7)	0.96 (0.64, 1.44)	0.85
	MSP2(FC27)	1.10 (0.80, 1.51)	0.55
May 2003[†]	AMA1(W2mef)	1.06 (0.79, 1.42)	0.70
	AMA1(HB3)	1.08 (0.80, 1.44)	0.63
	AMA1(3D7)	1.09 (0.80, 1.47)	0.59
	MSP2(3D7)	1.05 (0.70, 1.58)	0.80
	MSP2(FC27)	1.2 (0.83, 1.73)	0.34

Notes

[◊] Univariate poisson regression.

[Δ] Analysis of all samples (all 6 sampling points) with the addition of an indicator for those children contributing more than one observation to the analysis.

[†] Analysis covering the 6 month period subsequent to the cross sectional bleed indicated.

No analysis for October 2003, May 2004, and October 2004 cross-section bleeds due to lack of malaria episodes after the October 2003 sampling.

P-values ≤0.1 indicated in bold.

Univariate analysis of the association between antibody responses and risk of malaria using all the samples with the inclusion of a repeated sampling identifier gave different results to those from single sampling analysis. Antibody responses to AMA1(W2mef) were significantly associated with an increased risk of clinical malaria (IRR 1.46, 95% CI 1.23-1.72, $p < 0.001$) whereas those to AMA1(HB3) and AMA1(3D7) had no effect on the risk of clinical malaria (Table 5.2). Antibodies to MSP2(3D7) had a borderline association with protection from clinical malaria (IRR 0.77, 95% CI 0.59-1.01, $p = 0.06$) whereas those to MSP2(FC27) were somewhat associated with an increased risk of malaria though this was not significant (IRR 1.20, 95% CI 0.94-1.55, $p = 0.15$).

Table 5.3 The adjusted association between antibody responses to merozoite antigens and the risk of clinical malaria in the 6 months subsequent to sampling time

	Multivariable analysis[◊]	IRR (95% CI)	*P*-value
All samples[Δ]	AMA(W2mef)	1.54 (1.28, 1.85)	**<0.001**
	AMA(HB3)	1.1 (0.85, 1.43)	0.46
	AMA(3D7)	1.08 (0.83, 1.42)	0.57
	MSP2(3D7)	0.7 (0.5, 0.96)	**0.03**
	MSP2(FC27)	1.2 (0.9, 1.61)	0.22
May 2002[†]	AMA(W2mef)	1.12 (0.67, 1.87)	0.67
	AMA(HB3)	1.16 (0.69, 1.93)	0.57
	AMA(3D7)	1.14 (0.67, 1.94)	0.64
	MSP2(3D7)	0.38 (0.17, 0.86)	**0.02**
	MSP2(FC27)	1.36 (0.83, 2.22)	0.23
October 2002[†]	AMA(W2mef)	0.98 (0.81, 1.19)	0.85
	AMA(HB3)	0.92 (0.7, 1.22)	0.58
	AMA(3D7)	0.9 (0.67, 1.21)	0.49
	MSP2(3D7)	0.81 (0.52, 1.27)	0.36
	MSP2(FC27)	0.94 (0.65, 1.36)	0.73
May 2003[†]	AMA(W2mef)	1.01 (0.72, 1.4)	0.96
	AMA(HB3)	1.03 (0.74, 1.44)	0.85
	AMA(3D7)	1.05 (0.74, 1.48)	0.79
	MSP2(3D7)	1.04 (0.65, 1.66)	0.87
	MSP2(FC27)	1.23 (0.78, 1.94)	0.37

Notes

[◊] Multivariable poisson regression.

[Δ] Analysis of all samples (all 6 sampling points) with the addition of an indicator for those children contributing more than one observation to the analysis.

[†] Analysis covering the 6 month period subsequent to the cross sectional bleed indicated.

No analysis for October 2003, May 2004, and October 2004 cross-section bleeds due to lack of malaria episodes after the October 2003 sampling.

P-values ≤0.1 indicated in bold.

The adjusted risk of clinical malaria was investigated by multivariable analysis that included the factors mentioned earlier (age, parasitaemia, and haemoglobinopathies). For the single time point analysis, only antibody responses to MSP2(3D7) in May 2002 were significantly associated with protection from clinical malaria (adjusted IRR 0.38, 95% CI 0.17-0.86, $p = 0.02$) (Table 5.3). Though anti-MSP2(FC27) responses had been weakly associated with an increased risk of malaria in the univariate analysis, the weak association was lost in the multivariable analysis. None of the other antibody responses at all three sampling points were associated with an increased or decreased risk of malaria. When all the samples were used in the multivariable analysis along with the inclusion of a repeated sampling identifier, anti-AMA1(W2mef) antibodies were strongly associated with a higher risk of malaria (adjusted IRR 1.54, 95% CI 1.28-1.85, $p < 0.001$) (Table 5.3). In contrast, anti-MSP2(3D7) antibodies were associated with protection from clinical malaria

(adjusted IRR 0.70, 95% CI 0.5-0.96, p = 0.03). Antibody responses to AMA1(HB3), AMA1(3D7), and MSP2(FC27) had no significant effect on the risk of malaria.

5.3.3 Longitudinal analysis of factors affecting the risk of malaria

The associations between antibody responses and the risk of malaria in the six months after sampling – as described in the previous sections – was important for informing us when antibodies had their greatest effect on malaria risk and also allowed for comparison with published literature. In the single time point analysis the inclusion of a repeated sampling indicator permitted use of all the samples at once but did not take into account the chronological nature of the sampling or the interdependency of both the covariates and outcome. The best way to take all these into account was by using generalised estimating equations (GEE).

To assess the contribution of non-antibody factors on the risk of clinical malaria over the entire study period, univariate poisson GEE was carried out on age, concurrent parasitaemia and the haemoglobinopathies. Age had a minor effect on reducing the risk of malaria over the study period (IRR 0.93, 95% CI 0.88-0.98, p = 0.01) compared to the sickle trait which significantly reduced the risk of malaria to a much greater degree (IRR 0.41, 95% CI 0.24-0.70, p = 0.001) (Table 5.4). Those children who had concurrent parasitaemia at only one cross sectional bleed were at a 2-fold higher risk of malaria compared to those without any concurrent parasitaemia (IRR 2.05, 95% CI 1.56-2.69, $p < 0.001$). Concurrent parasitaemia at more than one cross sectional sampling point and alpha-thalassemia had no association with the risk of malaria.

Table 5.4 Factors affecting the risk of clinical malaria over the study

Univariate analysis[6]	IRR (95% CI)	*P*-value
Age (years)	0.93 (0.88, 0.98)	**0.01**
Parasitaemic once[1]	2.05 (1.56, 2.69)	**<0.001**
Recurrent parasitaemia[2]	0.95 (0.61, 1.5)	0.84
Sickle trait	0.41 (0.24, 0.7)	**0.001**
Alpha-thal HET[3]	0.95 (0.72, 1.26)	0.72
Alpha-thal HOM[3]	0.76 (0.51, 1.12)	0.17

Notes
[6] Univariate generalised estimating equation (GEE), exchangeable correlation structure.
All samples.
[1] Concurrent parasitaemia at one cross sectional bleed during the study.
[2] Concurrent parasitaemia at more than one cross sectional bleed during the study.
[3] Alpha-thalasssemia: HET = heterozygous, HOM = homozygous.
P-values ≤0.1 indicated in bold.

5.3.4 Contrasting associations between anti-merozoite antibody responses and protection against malaria

Univariate analysis of the association of antibody responses to AMA1 and MSP2 with the risk of clinical malaria was analysed by GEE with exchangeable correlation structure using all the samples in the cohort. Increasing antibody responses to AMA1(HB3), AMA1(3D7), MSP2(3D7)and MSP2(FC27) were not associated with risk of malaria over the study period (Table 5.5). Interestingly, antibody responses to AMA(W2mef) were significantly associated with an increased risk of malaria over the study period (IRR 1.56, 95% CI 1.34-1.80, $p < 0.001$).

Table 5.5 The effect of antibody responses to merozoite antigens on the risk of clinical malaria over the study

Univariate analysis[6]	IRR (95% CI)	*P*-value
AMA1(W2mef)	1.56 (1.34, 1.8)	**<0.001**
AMA1(HB3)	1.1 (0.91, 1.32)	0.34
AMA1(3D7)	1.07 (0.88, 1.31)	0.50
MSP2(3D7)	0.86 (0.64, 1.15)	0.32
MSP2(FC27)	1.15 (0.91, 1.46)	0.25

Notes
[6] Univariate generalised estimating equation (GEE), exchangeable correlation structure.
All samples.
P-values ≤0.1 indicated in bold.

Multivariable analysis of the protective associations of antibody responses to AMA1 and MSP2 that adjusted for the factors above was then performed. As seen in the univariate analysis, antibody

responses to AMA1(HB3) and AMA1(3D7) were not associated with the risk of clinical malaria (Table 5.6). Antibody responses to MSP2(3D7) were associated with protection though this effect was not statistically significant (IRR 0.88, 95% CI 0.62-1.23, p = 0.44) whereas those to MSP2(FC27) were associated with an increased risk of malaria (IRR 1.31, 95% CI 0.98-1.75, p = 0.07). As seen in the univariate analysis, antibody responses to AMA1(W2mef) were associated with a significantly increased risk of malaria over the study (IRR 1.75, 95% CI 1.47-2.09, $p <$ 0.001). Recent studies report that the removal of individuals without any exposure from analysis strengthened the protective associations of some merozoite antibodies against clinical disease (Bejon et al. 2009). Multivariable analysis was therefore repeated using only those children who had recorded exposure to *Plasmodium* over the study period. This analysis also showed that higher antibody responses to AMA1(W2mef) were associated with an increased risk of malaria (IRR 1.56, 95% CI 1.32-2.1.64, p <0.001) (Table 5.6). The protective effect of anti-MSP2(3D7) antibodies on clinical malaria was suggested by a reduced risk ratio of borderline statistical significance (IRR 0.75, 95% CI 0.55-1.04, p = 0.08) although the increased risk of malaria associated with anti-MSP2(FC27) responses was lost. Even with the removal of non-exposed children from the analysis, antibody responses to AMA1(HB3) and AMA1(3D7) had little association with the risk of clinical malaria.

Table 5.6 The adjusted effect of antibody responses to merozoite antigens on the risk of clinical malaria over the study period

Multivariable analysis[◊]	All children[Δ]		Exposed children[†]	
	IRR (95% CI)	*P*-value	IRR (95% CI)	*P*-value
AMA(W2mef)	1.75 (1.47, 2.09)	**<0.001**	1.56 (1.32, 1.84)	**<0.001**
AMA(HB3)	1.05 (0.84, 1.3)	0.68	1 (0.82, 1.22)	0.99
AMA(3D7)	1.04 (0.82, 1.31)	0.75	1.01 (0.81, 1.25)	0.94
MSP2(3D7)	0.88 (0.62, 1.23)	0.44	0.75 (0.55, 1.04)	**0.08**
MSP2(FC27)	1.31 (0.98, 1.75)	**0.07**	1.04 (0.79, 1.37)	0.79

Notes

[◊] Multivariable generalised estimating equation (GEE), exchangeable correlation structure.

[Δ] All samples.

[†] Only those children with recorded *P. falciparum* exposure during the study.

P-values ≤0.1 indicated in bold.

5.4 Discussion

This study is particularly effective in both highlighting and demonstrating the contrasting results that are reported in the published literature. Published studies have often taken the simplest approach to infer the protection accorded by antibodies to malaria antigens due to the logistics of setting up extended longitudinal studies. Those that have sampled antibody levels in a repeated longitudinal manner have either then not included all available data or have instead broken down the longitudinal data into a series of cross-sectional queries. This study is unique in the approaches used to investigate associations between antibodies and protective immunity using all available data. In order to compare this study with the published literature logistic regression was performed with clinical malaria as the outcome over a period of six months post sampling.

Analysis of the effect on malaria immunity of antibody responses measured at a single time point showed that it is possible to get different associations with protection or risk that are simply dependent on the time that the study is performed. As indicated in chapter 4, a number of children maintained a similar antibody profile to all AMA1 and MSP2 variants throughout the study period with minor fluctuations in their responses. Analysis suggests that a major contributor to the relationship between antibodies and protection lies in the level of malaria transmission during the period in question. Because one particular antibody response is protective over a certain period does not mean that the same response will be at a different time. Looking at the data in this 'snapshot' fashion allowed it to be compared with published literature as most association studies measured antibodies at baseline and then calculated protection over a define period (often six months). Results presented here suggest that using a single sample collection and follow-up to assess protective associations has its limitations. It is believed that the antibody response at any one time encompasses the capacity of an individual to respond as well as their response to previous malaria exposure and therefore makes single time point sampling a valid method (Drakeley et al. 2005). Contrary to this; the results discussed in previous chapters indicate that in children in this cohort, a large portion of the antibody response recorded at any one time is largely a response to antigen currently in or very recently in the system. This is likely to be due to declining malaria transmission levels.

The loss of statistical significance of the associations between MSP2 antibody responses and risk of malaria at some time-points could be due to declining malaria transmission. The falling malaria incidence then resulted in anti-MSP2(3D7) antibodies being associated with high reductions in risk which were not statistically significant. Had the incidence of malaria been higher in this cohort, these associations may have reached statistical significance. This limitation was addressed in part when all the samples were analysed together or when longitudinal analysis was used. Use of all the samples with an indicator of repeated sampling led to better use of the data available. By doing so, the model took into account that the same individuals can have different antibody responses and outcomes. What it could not encompass was that the measures were related to each other in time and therefore lost the impact of changing malaria transmission on both the antibody levels and the outcomes. This method tended to result in associations that were different when compared to those of the single-time point analysis. For example, the multivariate analysis in May 2002 showed that antibody responses to AMA(W2mef) were associated with an increased risk of malaria and yet this was not the case in October 2002 and May 2003. The results indicated that protection from clinical malaria in this cohort was both antigen and variant specific. Antibodies to AMA1 were significantly associated with an increased risk for the W2mef variant though this was not the case for the HB3 and 3D7 variants. Antibodies to MSP2 (3D7) were significantly associated with protection yet those for MSP(FC27) were associated with an increased risk of malaria though this was not statistically significant. The results from the combined cross sectional risk analysis are very similar to those seen in an earlier study carried out in the same area with regards to MSP2 (Polley et al. 2006). Antibodies to type A and type B MSP2 were analysed in a cohort of individuals 0-85 years of age in October 2000. The authors found that antibodies against type A MSP2 were associated with a reduced risk (OR = 0.87, p = 0.64) and those to type B with an increased risk of malaria in the 6 months post sampling (OR = 1.23, p = 0.48). These results are comparable to those seen in the study described here where antibodies to MSP2(3D7) were associated with a reduced risk (IRR 0.70, p = 0.03) and those to MSP2(FC27) with a higher risk (IRR = 1.20, p = 0.22) of malaria in the 6 months post sampling. Antibody responses to AMA1(3D7) had no effect on the risk of malaria in this study (IRR = 1.08, p = 0.57) although earlier studies in the same area showed that there could be a protective effect by anti-AMA1(3D7)

antibodies (RR = 0.73, $p = 0.075$) (Polley et al. 2004). The differences in the earlier study from the one described in this chapter included a wider age distribution, higher rates of malaria transmission, and possibly the inclusion of schizont extract responses in the multivariable analysis. Schizont extract was not included in the multivariable model in this study as it is not independent of the antibody responses being evaluated. As schizont extract proteins include both AMA1 and MSP2, it does not meet the definition of a confounder. Inclusion of schizont extract into the models used in this analysis had little effect on the associations found in this chapter. Both sickle trait and alpha-thalassemia were adjusted for in the multivariable models as they were independently associated with lower antibody levels and reduced risks of malaria.

The longitudinal model used not only took into account that the independent and dependent variables changed over time, it also accounted for any changes in covariates such as age or concurrent parasitaemia. Both the univariate and multivariable analysis indicated that antibodies to AMA1(W2mef) were significantly associated with an increased risk of clinical malaria over the study period (p <0.001). In both the univariate and multivariable GEE analysis antibodies to AMA1(HB3), AMA1(3D7), and MSP2(FC27) had no association with clinical disease. The only antibody response to be associated with protection from malaria was that to MSP2(3D7) when children without recorded *Plasmodium* exposure were removed from the analysis, though this association was of borderline significance (p = 0.08). The results from the longitudinal GEE analyses were in agreement with those from the GLM analyses with a clustering indicator. This agreement underscored the importance of multiple measurements when a dynamic variable such as antibody levels is being evaluated.

As indicated in previous chapters, antibody levels were also dependent on a number of dynamic factors that change with time, such as age, cumulative and recent malaria exposure, and malaria transmission levels. These changing covariates are intrinsically accounted for in longitudinal analysis with GEE. Following the earliest definitive studies that antibodies are involved in immunity to malaria, more recent immune-epidemiological studies often fail to discuss in depth the reasons that an antibody response could be associated with clinical disease, rather than protection. An explanation for this could be that the lack of information on exposure and/or parasitaemic or malaria episodes over a considerable period of time makes it difficult to assess a result that is

contrary to published results. Though it has been observed and is accepted that antibody levels are dynamic, the idea that the presence of antibodies to the same malarial antigen can indicate either protection or susceptibility in the same individuals is not widely explored.

Antibodies to any malarial antigen will only be induced on exposure. Some of these antibodies will protect against further infection from the same strain, or may contribute to protection against many strains due to the existence of conserved epitopes. Other antibodies will be directed against 'non-immunity' epitopes. That is, epitopes that even when bound to, will not affect the function of the antigen or parasite survival adversely. Every exposure and re-exposure results in a combination of all three types of antibody to each antigen. As cumulative exposure leads to immunity to malaria, it could be hypothesised that the majority of the initial antibody response is made up of those antibodies directed to 'non-immunity' epitopes and therefore act as a marker of disease/infection. Additionally, antibodies may need to reach a threshold concentration or affinity before they have significant activity against parasites in vivo. Each successive exposure to that particular antigen results in antibody response that is decreasingly made up of these 'non-immunity' antibodies and increasingly of 'immunity' antibodies. How fast or after how many exposures this occurs is still unknown and would be dependent on a number of factors that are dependent on both the host and the antigen in question. This idea may shed some light on the fact that antibodies to different malarial antigens are variously associated with protection from or risk of clinical malaria in the published literature. As it was not possible to investigate the infective genotype or phenotype of each infection in this study, I can only speculate. What this study did show was that malaria transmission will have an effect on inferred protection or risk associations. In this case, a reduction in transmission may have an effect on the number of circulating *P. falciparum* strains in the population. The reduction in strains would leave larger gaps in each individual's antibody repertoire therefore leading to the production of more 'non-immunity' antibody responses on exposure. This could explain how the high antibody response to AMA1(W2mef) seen in the population may reflect recent exposure to this antigen which appeared low before May 2002 (see chapter 4). This interpretation might explain why higher anti-AMA1(W2mef) antibodies were associated with an increased risk of disease in this study.

The finding that parasitaemia was longitudinally associated with increased risk suggests that these are the most exposed children. Indeed, the factor of differences in exposure has already been suggested in recent publications (Polley et al. 2004; Bejon et al. 2009). When exposure was factored into the analysis, the increased risk of disease with anti-AMA1(W2mef) antibodies remained significant. The removal of non-exposed children in the analysis led to a stronger association between antibodies to MSP2(3D7) and protection from disease which approached statistical significance. This result is also in agreement with the assessment made in the previous chapter. It was noted that 67.9% of the children who had concurrent parasitaemic once at cross-sectional bleeds had more than one incident of recorded parasitaemia in the intervals. This is a much larger proportion than those seen in the group who never had concurrent parasitaemia at sampling (23.7%) or those who had recurrent parasitaemia at sampling (32.5%). That children with high antibody responses to A4 schizont proteins were at an increased risk of malaria suggests that these children had greater *Plasmodium* exposure which translates to a higher risk of disease.

Only in the longitudinal analysis was it possible to see the small but significant effect that older age in childhood had on the reduction of malaria. Although the effect of age is commonly observed by falling rates of parasitaemia and severe illness, its effect has not been identified independently of cumulative exposure as was possible in this analysis. In both the single time point and the longitudinal analysis the sickle trait was associated with a reduced risk of malaria in this cohort. This is in agreement with published findings on this cohort as well as in other studies (Willcox et al. 1983; Hill et al. 1991; Aidoo et al. 2002; Williams et al. 2005a; Williams et al. 2005b). This immunity appears in part due to the physical nature of the erythrocytes as well as regulation of the immune response in HbAS children (Marsh et al. 1989; Bayoumi et al. 1990; Abu-Zeid et al. 1992; Williams et al. 2005a). In this study, alpha-thalassemia did not confer significant protection against malaria. This finding is also in agreement with published data on children from the same study area (Wambua et al. 2006).

A recent study carried out in Papua New Guinea showed that although antibodies to merozoite antigens were associated with protection from malaria they were not associated with protection from re-infection (Stanisic et al. 2009). Re-infection and parasite density thresholds are rarely used as an outcome in immune-epidemiological studies yet finding those antibody responses that protect

against them could be very important in identifying the particular antigens targeted in those individuals who maintain sub-patent infections. This study – with its intensive weekly surveillance – could have led to further insight into this process. Unfortunately as there was no active surveillance for parasitaemia – only for clinical illness – it was not possible to do so in the analysis. This study revealed that assessment of the protective effects of antibodies measured at a single time point assists in informing when the antibody response in question is exerting its greatest effect. This was the case for anti-MSP2(3D7) which were most protective in the six months following the May 2002 bleed. But it must be noted that without the other cross sectional bleeds to compare with, the interpretation of this association may have been incomplete. The advantage of the longitudinal method was that it allowed for a more complete picture of the role of antibody responses in protection against malaria. As malaria immunity is a dynamic process that is affected by a number of factors of which some are dynamic (such as ongoing malaria transmission and age) and others are static (such as haemoglobinopathies), analysis methods that include all these variables in their entirety are probably the best options for studying the role of anti-malarial antibodies in protection. Longitudinal sampling will need to be an important consideration in future studies. In this study, antibody responses to AMA1(W2mef) were associated with an increased risk of clinical disease whereas those to MSP2(3D7) were possibly associated with protection from clinical disease in children below the age of 10 years.

6 Acquisition and maintenance of isotype and subclass antibody responses to AMA1(3D7) and their association with protection against clinical malaria

6.1 Introduction

Functional differences may exist in antibodies of the same specificity and one reason for these differences may be subclasses. It was not until the early 1990s that differences in antibody isotypes and subclasses were reported in susceptible and protected individuals (Bouharoun-Tayoun and Druilhe 1992). In this study, immunoglobulin G1 (IgG1) and immunoglobulin G3 (IgG3) were the dominant antibody subclasses in protected individuals. IgG1 and IgG3 are cytophilic antibodies that mediate complement fixation and phagocytosis (Bredius et al. 1994). In the study described earlier; IgM – a low affinity antibody that is produced on primary exposure to antigen – predominated in younger children who were susceptible to disease (Bouharoun-Tayoun and Druilhe 1992). Further studies in Africa and Asia have also showed that IgG1 and IgG3 are the predominant antibody responses to merozoite antigens (Taylor et al. 1995; Shi et al. 1996; Rzepczyk et al. 1997; Polley et al. 2006; Nebie et al. 2008; Stanisic et al. 2009).

A number of factors including host genetics, antigen type, cumulative exposure, and age have been implicated in determining the predominant subclass response to merozoite antigens. An example of host genetic influence on subclass responses was a study carried out in Burkina Faso which showed that related individuals tended to display similar subclass antibody profiles (Aucan et al. 2001). Whereas the predominant IgG response to MSP2 is IgG3 in normal individuals, three studies showed that individuals with the sickle trait (HbAS) had measurable IgG2 responses to MSP2 in comparison to their normal (HbAA) counterparts (Aucan et al. 2000; Ntoumi et al. 2002; Ntoumi et al. 2005). The type of *Plasmodium* exposure that individuals experience may also have an effect as shown by the *in vitro* stimulation of peripheral blood mononuclear cells from Senegalese adults with complete parasite extract that resulted in a mixed IgG subclass response whereas stimulation with a single recombinant antigen always resulted in a single IgG subclass

response (Garraud et al. 2002). Studies carried out in Senegal and Gambia indicated that there was an exposure-related preponderance for IgG1 or IgG3 responses to MSP1 and MSP2 but these results remained unconfirmed in a later Brazilian study (Nguer et al. 1997; Taylor et al. 1998; Scopel et al. 2005). Increasing age has also been suggested to polarize the antibody response to IgG3 for MSP1 and MSP2, and to IgG1 for AMA1 though this was not seen in a more recent study (Scopel et al. 2006; Tongren et al. 2006; Stanisic et al. 2009). In fact, this recent treatment re-infection study that compared the subclass response to different AMA1 and MSP2 variants in children showed that the main factor influencing the subclass response was the antigen rather than the host (Stanisic et al. 2009).

Anti-AMA1 antibodies in malaria endemic populations are predominantly IgG1 and IgG3 with very little IgG2 or IgG4 detected (Riley et al. 2000; Polley et al. 2004; Metenou et al. 2007; Nebie et al. 2008; Stanisic et al. 2009). IgG1 responses predominated over IgG3 responses both in prevalence and intensity (Riley et al. 2000; Nebie et al. 2008), but titres of both increased with age and exposure to malaria (Stanisic et al. 2009). To date, there have been no studies examining the longitudinal acquisition and maintenance of subclass antibodies to AMA1 or other merozoite antigens.

It has been suggested that age and exposure skews the antibody response to AMA1 towards a predominantly IgG1 response. The implication here is that at a young age both IgG1 and IgG3 are similarly induced. The structure of this study allows for investigation into the balance of IgG1 and IgG3 responses over time. Here, I describe the patterns of subclass (IgG1, IgG3) and isotype (IgG, IgM) antibody responses to AMA1(3D7) and compare the effects of various factors (host genetics, cumulative exposure, and age) on the magnitude of the antibody response. In particular, this chapter examines whether repeated exposure or increasing age leads to changes in the pattern of IgG subclass responses and investigates whether specific isotype or subclass responses to AMA1(3D7) are associated with protection against clinical malaria in this cohort of children.

6.2 Methods

6.2.1 Enzyme-linked immunosorbent assay (ELISA)

IgG1, IgG3 and IgM antibody levels to AMA1(3D7) were measured in all samples by high throughput ELISA. The details of this assay can be found in chapter two but will be described briefly here. Nunc Maxisorp 384 well plates (Nunc) were coated with 50µl per well of AMA1(3D7) diluted in phosphate buffered saline (PBS) at a concentration of 0.5µg/ml. The plates were sealed and incubated overnight at 4°C. Plates were then washed twice using PBS with 0.25% Tween 20 (wash buffer). Non-specific binding was blocked by addition of 100µl per well of PBS with 0.1% Casein (Pierce) and 0.05% Tween 20. The plates were then sealed and incubated overnight at 4°C. The plates were washed once before addition of sera. 50µl of sera diluted in PBS with 0.1% Casein and 0.05% Tween 20 was added to each well and incubated for 2 hours at room temperature. IgG1, IgG3, and IgM were measured at a sera concentration of 1:100. After the sera incubation, the plates were washed five times using wash buffer. Detection of IgM was done using 50µl of a 1:2500 dilution of HRP-conjugated polyclonal goat anti-human IgM (Chemicon, Melbourne Australia) in PBS with 0.1% Casein and 0.05% Tween 20 as the secondary antibody. HRP-conjugated monoclonal mouse anti-human IgG (IgG1 clone HP6069A, Calbiochem-Novabiochem Corp, CA USA) and HRP-conjugated monoclonal mouse anti-human IgG3 (IgG3 clone HP6047, Calbiochem-Novabiochem Corp, CA USA) were also used at 1:2500 dilutions to detect IgG1 and IgG3 responses to AMA1(3D7). The plates were then washed five times in wash buffer and 50µl of ABTS (A3219, Sigma) was added to each well. The plates were incubated for 30 minutes in the dark to allow for sufficient colour change. Addition of 50µl of 1% SDS per well stopped the reaction and absorbance was read at 405nm using an Envision plate reader (Perkin Elmer). Antibody levels were expressed as an absorbance value (OD).

6.2.2 Statistical Analysis

All data analyses were performed with STATA version 9.2 (StataCorp, College Station, TX USA). Statistical analysis was carried out as described in detail in chapters 2 – 5 and will only be described briefly here. All samples were used in the analyses unless stated. Significance was reached at the 0.05 level for all tests. The chi^2 test for trend was used to assess the acquisition of antibodies in different age groups, and the Kruskal-Wallis test to compare antibody levels. The probability of a high antibody response (above the 75^{th} centile) after recorded exposure to *P. falciparum* was estimated by logistic regression, fitting the number of recorded parasitaemic episodes in the 6 months prior to sample time as a factor and adjusting for age. The logits from these models were converted into probabilities to give estimates of risk and then plotted using the lowess smoothing technique. This same analysis was employed to estimate and plot the probability of being sero-positive. The association between antibody responses to AMA1(3D7) and the risk of malaria was investigated by two methods which are described in detail in chapters 2 and 5. Univariate and multivariable generalised linear models (GLM) were used to investigate the relationship between antibody levels at a single time point ant the risk of malaria in the subsequent six months. Univariate and multivariable generalised estimating equations (GEE) were used to investigate relationship between antibody levels at several time points and the risk of malaria over the entire study period.

6.3 Results

6.3.1 The predominant IgG response to AMA1 in Ngerenya is of the IgG1 subclass and is associated with age and active parasitaemia

Using cut-off values determined by a panel of negative controls, the acquisition of different isotype and subclass antibody responses to AMA1(3D7) was investigated by examining the prevalence of total IgG, IgG1, IgG3 and IgM. In all age groups (in one year intervals), sero-positivity ranged widely with the lowest prevalence seen for IgM followed by IgG3 responses, and highest for IgG1.

Prevalence of sero-positivity of all antibodies increased with age following a decline in maternal antibodies from the age of one year (Figure 6.1).

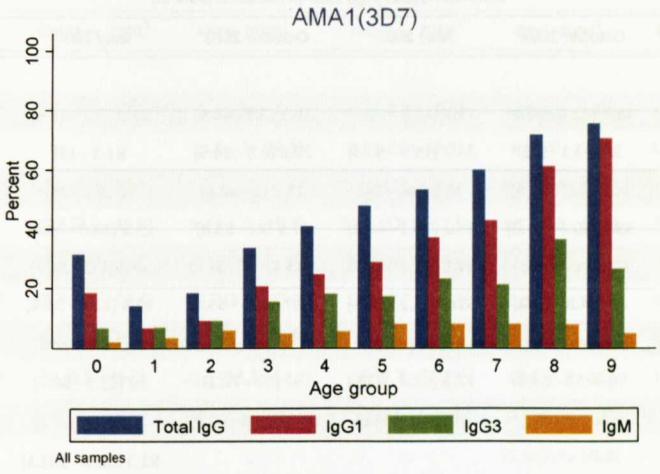

Figure 6.1 Proportion of samples with detectable antibody responses to AMA1(3D7) by age group. All samples in the study were included, defined by age at sampling.

By the age of one year, when all maternal antibodies have disappeared from the child's circulation, only 8.3% (95% CI: -3% - 19.7%) of children tested positive for IgM in May 2004 (Table 6.4), compared with 34.7% (95% CI: 15.9% - 53.4%) for total IgG in May 2003 (Table 6.1). In comparison with the lower prevalence of IgM responses, 15.4% (95% CI: 1.2% – 29.6%) and 12.5% (95% CI: -1.1% - 26.1%) of one year olds had IgG1 and IgG3 antibody responses to AMA1(3D7) in May 2003 and May 2004 respectively (Tables 6.2 and 6.3). By the age of 8 years, all children had total IgG and IgG1 responses to AMA1 in October 2002 (Tables 6.1 and 6.2). This was never the case for IgG3 and IgM which had maximal prevalence values of 51.5% (95% CI: 34.1% - 68.9%) and 12.1% (95% CI: 0.8% - 23.5%) in October 2003 in children above 7 years of age (Tables 6.3 and 6.4). For all three IgG responses tested (total IgG, IgG1 and IgG3), there was a significant increase ($p < 0.01$, chi^2 test for trend) in the prevalence of responses with age at all cross sectional bleeds (Tables 1 – 3). Only during the May 2002 cross sectional bleed was there a significant increase ($p < 0.01$, chi^2 test for trend) in the prevalence of IgM responses with age (Table 6.4).

Table 6.1 Prevalence of detectable IgG responses to AMA1 (3D7) by age and cross sectional bleed in the Ngerenya cohort

Age (years)	Anti-AMA1(3D7) IgG sero-prevalence (95% CI)						
	May 2002[1]	October 2002[1]	May 2003[1]	October 2003[1]	May 2004[1]	October 2004[1]	*P*-value[2]
0	23.4 (7.9 - 38.8)	48.7 (32.3 - 65.1)	29.7 (12.1 - 47.3)	26.1 (7.7 - 44.6)	23.9 (5.1 - 42.6)	26.7 (3.4 - 50)	0.384
1	8.4 (0.4 - 16.3)	3.2 (-3.1 - 9.3)	34.7 (15.9 - 53.4)	20.6 (6.8 - 34.5)	8 (-3 - 19)	12.5 (-1.1 - 26.1)	0.386
2	35.9 (20.6 - 51.3)	20.6 (7.7 - 33.5)	13.7 (3.4 - 24)	18.8 (5 - 32.6)	13 (0.9 - 25)	6.3 (-2.4 - 14.9)	**0.002**
3	46.4 (30.9 - 61.9)	35.9 (20.6 - 51.3)	28.2 (12.3 - 44.1)	37.9 (22 - 53.8)	29.8 (14.8 - 44.8)	17.9 (3.4 - 32.4)	**0.027**
4	40.7 (23.3 - 58)	63.4 (45.8 - 81)	47.5 (31.8 - 63.3)	40.5 (25.4 - 55.6)	17.3 (3.2 - 31.3)	33.4 (16.1 - 50.6)	**0.017**
5	63.9 (49.9 - 77.8)	49 (34.8 - 63.2)	51.8 (33.2 - 70.4)	46.2 (26.6 - 65.8)	39.4 (22.4 - 56.4)	30.4 (14.4 - 46.3)	**0.003**
6	71.1 (56.4 - 85.8)	55.2 (36.7 - 73.7)	50 (33.4 - 66.7)	57.2 (42 - 72.4)	41.7 (21.5 - 62)	33.4 (14 - 52.7)	**0.004**
7	65.3 (45.3 - 85.3)	68.6 (53 - 84.3)	77.5 (62.4 - 92.5)	52 (32 - 72.1)	50 (33.9 - 66.2)	50 (33.9 - 66.2)	**0.022**
8[6]	-	100 (0 - 0)	75 (55.5 - 94.6)	87.9 (76.6 - 99.3)	76 (58.9 - 93.2)	41.7 (21.5 - 62)	**0.003**
9[6]	-	-	-	-	81.3 (61.5 - 101.1)	72 (54 - 90.1)	0.506
P-value[3]	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	

Notes

[1] Cross sectional bleed.

[2] *P*-values calculated using a chi^2 test for trend for IgG prevalence in the same age group at different cross sectional bleeds (*P*-values ≤ 0.05 indicated in bold type).

[3] *P*-values calculated using a chi^2 test for trend for IgG prevalence in different age groups at the same cross sectional bleed (*P*-values ≤ 0.05 indicated in bold type).

[6] Missing values are due to a lack of children in that age group.

All samples included in analysis.

Table 6.2 Prevalence of detectable IgG1 responses to AMA1 (3D7) by age and cross sectional bleed in the Ngerenya cohort

Age (years)	Anti-AMA1(3D7) IgG1 sero-prevalence (95% CI)						
	May 2002[1]	October 2002[1]	May 2003[1]	October 2003[1]	May 2004[1]	October 2004[1]	*P*-value[2]
0	16.7 (3 - 30.3)	18.9 (6.1 - 31.8)	25.9 (9 - 42.8)	17.4 (1.5 - 33.3)	9.5 (-3.4 - 22.4)	20 (-1 - 41)	0.759
1	6.3 (-0.7 - 13.2)	3.1 (-3 - 9.3)	15.4 (1.2 - 29.6)	5.9 (-2.2 - 13.9)	4 (-3.9 - 11.9)	4.2 (-4 - 12.4)	0.761
2	20.5 (7.6 - 33.4)	10.3 (0.6 - 19.9)	2.3 (-2.2 - 6.7)	9.4 (-0.9 - 19.7)	3.2 (-3.1 - 9.6)	6.3 (-2.3 - 14.8)	**0.028**
3	26.8 (13 - 40.6)	12.8 (2.1 - 23.5)	21.9 (7.3 - 36.5)	27 (12.5 - 41.6)	18.9 (6.1 - 31.8)	14.3 (1 - 27.5)	0.536
4	28.1 (12.2 - 44)	20 (5.4 - 34.6)	30 (15.6 - 44.4)	33.3 (18.8 - 47.8)	13.8 (1 - 26.6)	16.7 (3 - 30.3)	0.289
5	36.2 (22.2 - 50.1)	24.5 (12.3 - 36.7)	27.6 (11 - 44.2)	30.8 (12.6 - 48.9)	33.3 (16.9 - 49.7)	21.2 (7 - 35.4)	0.452
6	57.9 (41.9 - 73.9)	37.9 (19.9 - 56)	36.1 (20.1 - 52.1)	35.7 (21 - 50.4)	20.8 (4.2 - 37.5)	25 (7.2 - 42.8)	**0.003**
7	47.8 (26.9 - 68.8)	48.6 (31.7 - 65.4)	58.1 (40.3 - 75.8)	32 (13.3 - 50.7)	39.5 (23.7 - 55.3)	34.2 (18.9 - 49.6)	0.089
8[6]	-	100 (0 - 0)	70 (49.3 - 90.7)	78.8 (64.6 - 93)	64 (44.7 - 83.3)	25 (7.2 - 42.8)	**<0.001**
9[6]	-	-	-	-	75 (53 - 97)	68 (49.3 - 86.7)	0.635
P-value[3]	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	

Notes

[1] Cross sectional bleed.

[2] *P*-values calculated using a chi^2 test for trend for IgG prevalence in the same age group at different cross sectional bleeds (*P*-values ≤ 0.05 indicated in bold type).

[3] *P*-values calculated using a chi^2 test for trend for IgG prevalence in different age groups at the same cross sectional bleed (*P*-values ≤ 0.05 indicated in bold type).

[6] Missing values are due to a lack of children in that age group.

All samples included in analysis.

Table 6.3 Prevalence of detectable IgG3 responses to AMA1 (3D7) by age and cross sectional bleed in the Ngerenya cohort

Age (years)	Anti-AMA1(3D7) IgG3 sero-prevalence (95% CI)						
	May 2002[1]	October 2002[1]	May 2003[1]	October 2003[1]	May 2004[1]	October 2004[1]	*P*-value[2]
0	10 (-1 - 21)	8.1 (-0.8 - 17.1)	3.7 (-3.6 - 11)	4.3 (-4.2 - 12.9)	4.8 (-4.6 - 14.1)	6.7 (-6.5 - 19.8)	0.436
1	6.3 (-0.7 - 13.2)	-	11.5 (-1 - 24.1)	8.8 (-0.9 - 18.5)	4 (-3.9 - 11.9)	12.5 (-1.1 - 26.1)	0.316
2	17.9 (5.7 - 30.2)	12.8 (2.1 - 23.5)	-	15.6 (2.8 - 28.5)	6.5 (-2.4 - 15.3)	-	**0.020**
3	24.4 (11 - 37.8)	10.3 (0.6 - 19.9)	9.4 (-0.9 - 19.7)	32.4 (17.1 - 47.8)	8.1 (-0.8 - 17.1)	3.6 (-3.5 - 10.6)	0.108
4	25 (9.7 - 40.3)	23.3 (7.9 - 38.8)	10 (0.5 - 19.5)	28.6 (14.7 - 42.5)	17.2 (3.2 - 31.3)	3.3 (-3.2 - 9.9)	0.085
5	23.4 (11.1 - 35.7)	18.4 (7.4 - 29.4)	20.7 (5.6 - 35.8)	19.2 (3.7 - 34.7)	12.1 (0.8 - 23.5)	9.1 (-0.9 - 19.1)	0.080
6	39.5 (23.7 - 55.3)	24.1 (8.2 - 40.1)	25 (10.6 - 39.4)	21.4 (8.8 - 34)	8.3 (-3 - 19.7)	12.5 (-1.1 - 26.1)	**0.003**
7	30.4 (11.1 - 49.7)	20 (6.5 - 33.5)	35.5 (18.3 - 52.7)	16 (1.3 - 30.7)	18.4 (5.9 - 31)	13.2 (2.2 - 24.1)	0.081
8[6]	-	-	50 (27.4 - 72.6)	51.5 (34.1 - 68.9)	32 (13.3 - 50.7)	12.5 (-1.1 - 26.1)	**0.005**
9[6]	-	-	-	-	31.3 (7.7 - 54.8)	24 (6.8 - 41.2)	0.614
P-value[3]	**0.001**	**0.009**	**<0.001**	**0.006**	**0.001**	**0.008**	

Notes
[1] Cross sectional bleed.
[2] *P*-values calculated using a chi^2 test for trend for IgG prevalence in the same age group at different cross sectional bleeds (*P*-values ≤ 0.05 indicated in bold type).
[3] *P*-values calculated using a chi^2 test for trend for IgG prevalence in different age groups at the same cross sectional bleed (*P*-values ≤ 0.05 indicated in bold type).
[6] Missing values are due to a lack of children in that age group.
All samples included in analysis.

Table 6.4 Prevalence of detectable IgM responses to AMA1 (3D7) by age and cross sectional bleed in the Ngerenya cohort.

Age (years)	Anti-AMA1(3D7) IgM sero-prevalence (95% CI)						
	May 2002[1]	October 2002[1]	May 2003[1]	October 2003[1]	May 2004[1]	October 2004[1]	*P*-value[2]
0	3.3 (-3.2 - 9.9)	2.7 (-2.6 - 8)	-	4.3 (-4.2 - 12.9)	-	-	0.416
1	2.1 (-2 - 6.2)	3.1 (-3 - 9.3)	3.8 (-3.7 - 11.4)	-	4 (-3.9 - 11.9)	8.3 (-3 - 19.7)	0.327
2	-	7.7 (-0.8 - 16.2)	4.5 (-1.7 - 10.8)	12.5 (0.8 - 24.2)	3.2 (-3.1 - 9.6)	6.3 (-2.3 - 14.8)	0.382
3	12.2 (2 - 22.4)	2.6 (-2.5 - 7.6)	3.1 (-3 - 9.3)	2.7 (-2.6 - 8)	2.7 (-2.6 - 8)	3.6 (-3.5 - 10.6)	0.107
4	9.4 (-0.9 - 19.7)	10 (-1 - 21)	5 (-1.9 - 11.9)	2.4 (-2.3 - 7.1)	6.9 (-2.5 - 16.3)	-	0.081
5	4.3 (-1.6 - 10.1)	6.1 (-0.7 - 12.9)	3.4 (-3.3 - 10.2)	19.2 (3.7 - 34.7)	12.1 (0.8 - 23.5)	6.1 (-2.2 - 14.4)	0.241
6	15.8 (4 - 27.6)	6.9 (-2.5 - 16.3)	5.6 (-2.1 - 13.2)	7.1 (-0.8 - 15.1)	4.2 (-4 - 12.4)	4.2 (-4 - 12.4)	0.088
7	26.1 (7.7 - 44.5)	8.6 (-0.9 - 18)	9.7 (-0.9 - 20.3)	4 (-3.9 - 11.9)	2.6 (-2.5 - 7.8)	2.6 (-2.5 - 7.8)	**0.002**
8[6]	-	-	5 (-4.8 - 14.8)	12.1 (0.8 - 23.5)	8 (-2.9 - 18.9)	4.2 (-4 - 12.4)	0.744
9[6]	-	-	-	-	6.3 (-6.1 - 18.6)	4 (-3.9 - 11.9)	0.747
P-value[3]	**0.001**	0.446	0.415	0.170	0.562	0.479	

Notes
[1] Cross sectional bleed.
[2] *P*-values calculated using a chi^2 test for trend for IgG prevalence in the same age group at different cross sectional bleeds (*P*-values ≤ 0.05 indicated in bold type).
[3] *P*-values calculated using a chi^2 test for trend for IgG prevalence in different age groups at the same cross sectional bleed (*P*-values ≤ 0.05 indicated in bold type).
[6] Missing values are due to a lack of children in that age group.
All samples included in analysis.

From 2 – 8 years of age, there was a significant decline in the prevalence of total IgG responses to AMA1 as malaria transmission declined ($p < 0.05$, chi^2 test for trend) (Table 6.1). This has already

been discussed in chapter 3. In comparison, the decline in malaria transmission only appeared to affect the prevalence of IgG1 and IgG3 responses in 2-, 6-, and 8-year olds ($p < 0.05$, chi^2 test for trend) (Tables 2 and 3). Only in 7-year olds was declining malaria transmission accompanied by a significant decline ($p < 0.01$, chi^2 test for trend) in the prevalence of IgM responses (Table 6.4).

In agreement with previous findings, total IgG responses were strongly associated with those of IgG1 at every cross sectional bleed (Table 6.5, Appendix 3 Tables 1-5). Correlation coefficients ranged from 0.67 in May 2003 (Appendix 3 Table 2) to 0.91 in October 2002 and May 2003 (Appendix 3 Tables 1 and 3) ($p < 0.001$, Spearman rank correlation). IgG3 responses were not as strongly associated with total IgG with correlation coefficients ranging from 0.49 in May 2003 (Appendix 3 Table 2) to 0.64 in May 2002 ($p < 0.001$, Spearman rank correlation) (Table 6.5). IgM responses were weakly associated with total IgG responses at all bleeds with correlation coefficients never rising above 0.4. IgM responses were most strongly associated with those of IgG3, with a correlation coefficient of 0.6 in May 2002 and October 2004 ($p < 0.001$, Spearman rank correlation) (Table 6.5, Appendix 3 Table 5). The weakest association between anti-AMA1 and anti-schizont extract protein responses was the IgM response. The correlation coefficient ranged from a low of 0.01 ($p = 0.89$, Spearman rank correlation) in May 2004 to 0.28 ($p < 0.001$, Spearman rank correlation) in May 2002 (Table 6.5, Appendix 3 Table 4). Of the IgG subclass responses, IgG1 had the strongest association with responses to schizont extract protein. Interestingly, there was a negative association between schizont extract responses and IgG responses during the May 2004 cross sectional bleed that was significant for total IgG and IgG1 ($p < 0.01$, Spearman rank correlation) (Appendix 3, Table 4).

Table 6.5 Correlations between antibody responses to AMA1(3D7) and schizont extract for the May 2002 cross sectional bleed.

	May 2002 Antibody responses				
	Schizont extract	Total IgG†	IgG1†	IgG3†	IgM†
Total IgG†	0.72*	-	-	-	-
IgG1†	0.61*	0.88*	-	-	-
IgG3†	0.56*	0.64*	0.74*	-	-
IgM†	0.28*	0.35*	0.5*	0.6*	-

Notes
Spearman rank correlation coefficients for IgG responses.
All samples tested.
† Antibody response to AMA1(3D7).
* *P*-value <0.001

The magnitude of the IgG isotype and subclass response to AMA1 was analysed in four age groupings (children less than a year, 1-3 yearls, 4-6 years, and 7-10 years) as well as by concurrent parasiemia at each cross sectional bleed. IgG1, IgG3, and IgM responses increased with age during the May 2002 cross sectional bleed in all children as well as aparasitaemic children ($p < 0.001$, Kruskal Wallis test), with children ages 7-10 years having significantly higher antibody responses than those aged 1-3 years ($p \leq 0.05$, Wilcoxon rank sum test) (Table 6.6). Though this remained the case for all the responses in May 2002, it was not the same at all cross sectional bleeds for IgG3 and IgM. Antibody responses in aparasitaemic children significantly increased with age ($p < 0.001$, Kruskal Wallis test) only in May 2002, May 2003, and May 2004 for IgG3 and IgM compared to all cross sectional bleeds for IgG1 (Appendix 3 Tables 6-8). In children with concurrent parasitaemia at sampling, antibody responses were similar in all age groups at all cross sectional bleeds. The only exceptions were for IgG1 in May 2003 and IgM in October 2003 where antibody responses significantly increased with age ($p < 0.001$, Kruskal Wallis test) (Appendix 3, Tables 7 and 8). Although concurrent parasitaemia increased the magnitude of the response to AMA1(3D7) with regards to all antibodies measured (all samples pooled regardless of age; $p<0.001$ Wilcoxon rank test), its effect was different for the different antibodies measured. A marked 16-fold and 13-fold increase in median antibody levels was observed for total IgG and IgG1, whereas a less marked 7-fold increase was noted for IgG3. In comparison to the effect on

IgG titres, there was only a modest 2-fold increase in the median IgM response among children with the presence of circulating parasites.

Total IgG levels to schizont protein extract were measured as a proxy for exposure to blood stage *P. falciparum* and responses were divided into tertiles. The antibody response to AMA1(3D7) was then analysed with regards to these categories. All four antibody responses measured (total IgG, IgG1, IgG3, and IgM) in May 2002 increased in magnitude with increasing exposure to *P. falciparum* and showed marked differences in those with high exposure compared to those with low and medium exposure (Figure 6.2). The IgG3 response was of a lower magnitude compared to the response to IgG1 although there was also a marked increase in the response of those with high prior exposure compared to those with less exposure (Figure 6.2C). Not only was there a very low magnitude IgM response to AMA1(3D7), the median response in those with the highest prior exposure was not markedly different from those with less prior exposure (Figure 6.2D). Analysis at each cross sectional bleed revealed a similar pattern except for IgG3 in May 2004 and IgM in both October 2002 and May 2004 (Appendix 3 Tables 11 and 12).

Table 6.6 Antibody responses to AMA1(3D7) by age group and parasite status at the May 2002 cross sectional bleed

May 2002 cross sectional bleed		All Samples					Aparasitaemic					Parasitaemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]
Total IgG[1]	N[2]	30	128	117	23		30	109	93	15		-	19	24	8	
	Median	0.03	0.02	0.18	0.18	**<0.001***	0.03	0.00	0.11	0.32	**<0.001***	-	0.48	0.60	1.11	0.769
	IQR	(0 - 0.08)	(-0.01 - 0.14)	(0.04 - 0.82)	(0.07 - 1.77)		(0 - 0.08)	(-0.02 - 0.06)	(0.03 - 0.61)	(0.07 - 1.48)		-	(0.06 - 1.7)	(0.22 - 1.66)	(0.07 - 2.15)	
IgG1[1]	N[2]	30	128	117	23		30	109	93	15		-	19	24	8	
	Median	0.01	0.01	0.16	0.16	**<0.001***	0.01	-0.01	0.11	0.29	**<0.001***	-	0.36	0.68	0.36	0.638
	IQR	(-0.03 - 0.19)	(-0.06 - 0.16)	(0.01 - 1.07)	(0.05 - 1.79)		(-0.03 - 0.19)	(-0.06 - 0.09)	(0 - 0.56)	(0.05 - 1.79)		-	(0.01 - 1.45)	(0.19 - 1.4)	(0.1 - 1.78)	
IgG3[1]	N[2]	30	128	117	23		30	109	93	15		-	19	24	8	
	Median	0.01	0.01	0.02	0.02	**<0.001***	0.01	0.01	0.02	0.03	**<0.001***	-	0.10	0.07	0.05	0.243
	IQR	(0 - 0.03)	(0 - 0.04)	(0.01 - 0.1)	(0.02 - 0.1)		(0 - 0.03)	(0 - 0.03)	(0.01 - 0.07)	(0.02 - 0.1)		-	(0.05 - 0.2)	(0.01 - 0.11)	(0.03 - 0.08)	
IgM[1]	N[2]	30	128	117	23		30	109	93	15		-	19	24	8	
	Median	0.02	0.02	0.05	0.05	**<0.001***	0.02	0.02	0.04	0.07	**<0.001***	-	0.06	0.06	0.08	0.578
	IQR	(0 - 0.05)	(0 - 0.06)	(0.02 - 0.08)	(0.02 - 0.16)		(0 - 0.05)	(0 - 0.05)	(0.02 - 0.08)	(0.02 - 0.13)		-	(0.03 - 0.1)	(0.02 - 0.11)	(0.04 - 0.17)	

Notes

[1] AMA1(3D7)-specific antibody response.

[2] Number of samples tested by ELISA.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

* *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

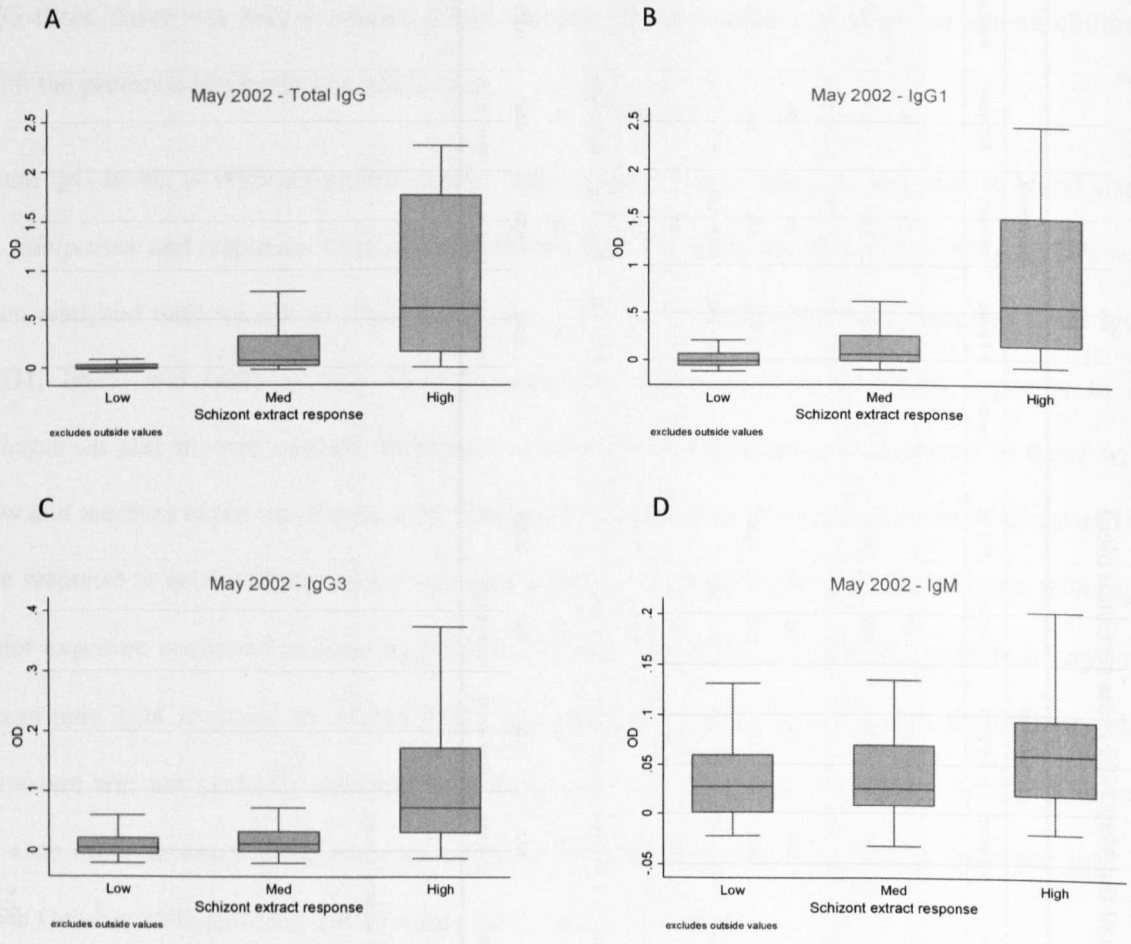

Figure 6.2 Magnitude of the antibody response to *P. falciparum* AMA1(3D7) by reactivity to A4 schizont protein. Representative graphs showing isotype and subclass antibody levels at the May 2002 cross sectional bleed. All samples tested by ELISA. Kruskal-Wallis test for equality of medians; $p<0.001$ for all antigens. Wilcoxon rank sum test for equality of median responses between two categories all $p<0.05$ except between 'Low' and 'Med' IgM response. (A) Total IgG, (B) IgG1, (C) IgG3, (D) IgM.

6.3.2 Lower IgG subclass responses are seen among children with alpha-thalassemia

Due to the protective effect of both the sickle trait and alpha-thalassemia on clinical malaria, the antibody response to AMA1(3D7) was investigated in children with different erythrocyte polymorphisms (Figure 6.3 Appendix 3 Tables 13-16). As previously reported in chapter 3, although total IgG titres in children with the sickle trait (HbAS) were generally lower than in their normal (HbAA) counterparts, this difference was only significant during the May 2003 cross sectional bleed ($p = 0.02$; Kruskal-Wallis test) (Appendix 3 Table 13). Although the overall range of IgG1 responses was wider in HbAA children, there was no difference in the median response when compared to that in HbAS children at all cross sectional bleeds (Figure 6.3B, Appendix 3

Table 14). Both HbAA and HbAS children had similar ranges of IgG3 responses without differences in their median values (Figure 6.3C, Appendix 3 Table 15). This observation was no different from that for IgM responses to AMA1(3D7) (Figure 6.3D, Appendix 3 Table 16). Also previously reported in chapter 3 was the lower total IgG response in alpha-thalassemia homozygotes as compared to heterozygote and normal children in October 2002, May 2003, and May 2004 ($p \leq 0.05$; Kruskal Wallis test) (Appendix 3 Table 13). The IgG1 response in alpha-thalassemia homozygotes was also significantly lower than that in the heterozygotes in October 2002 and May 2003 ($p \leq 0.05$; Wilcoxon rank test), and between homozygotes and normal children in Ocotber 2003 and May 2004 ($p \leq 0.05$; Wilcoxon rank test) (Appendix 3 Table 14). There was no difference in IgG3 responses in alpha-thalassemia homozygotes and heterozygotes compared to normal children at all cross sectional bleeds (Appendix 3 Table 15). Only in May 2003 was there a significantly lower IgM response in alpha-thalssemia homozygotes compared to the heterozygotes and normal children (Appendix 3 Table 16).

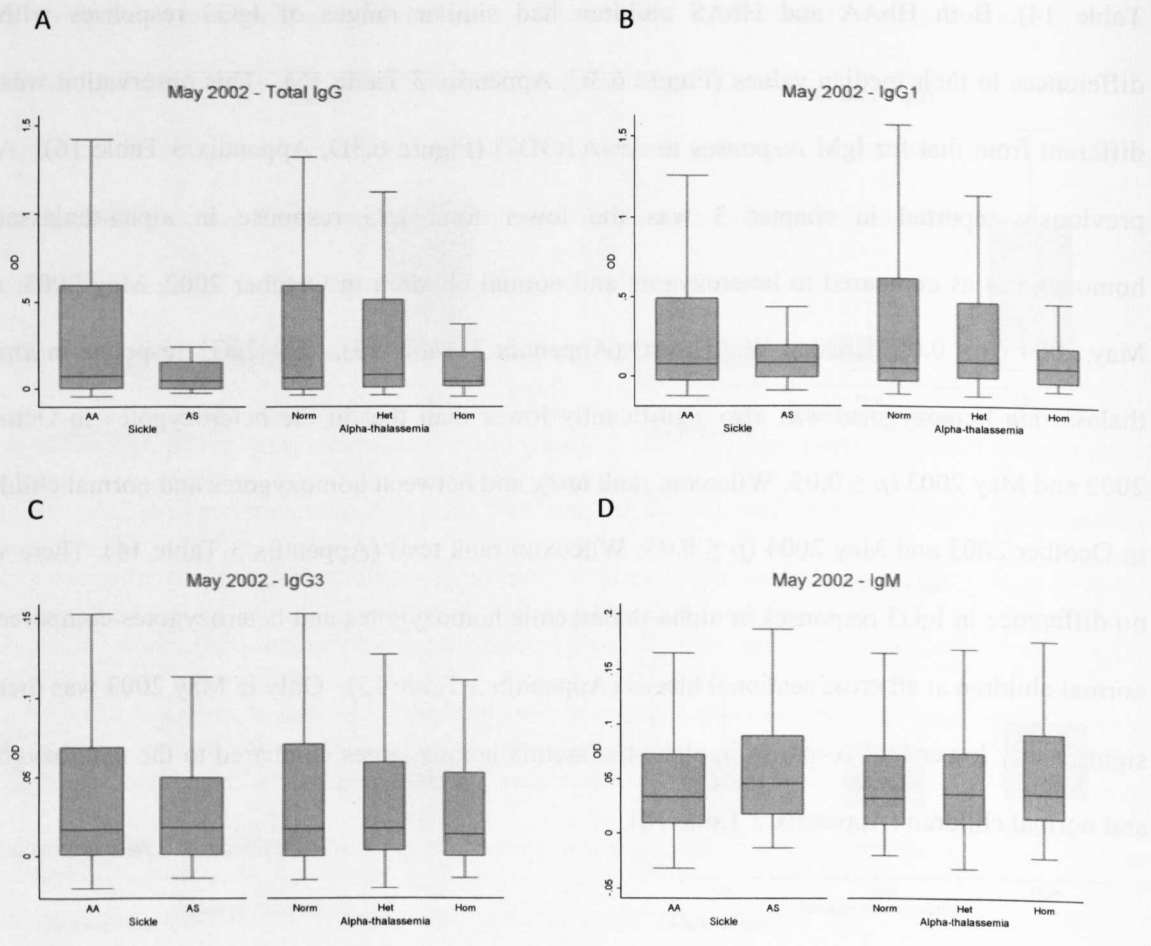

Figure 6.3 Magnitude of the antibody (IgG) response to *P. falciparum* merozoite antigens in the presence or absence of haemoglobinopathies (sickle trait and α-thalassemia). AA=Normal, AS=Sickle trait, Norm=Normal, Het=Heterozygous, Hom=Homozygous. Representative graphs showing IgG levels at the May 2002 cross sectional bleed. All samples tested by ELISA. Kruskal-Wallis test for equality of median responses in sickle trait and α-thalassemia categories non-significant for all antibody responses tested. Wilcoxon rank sum test for equality of median responses between two sickle trait categories non-significant for all antibody responses tested. (A) Total IgG, (B) IgG1, (C) IgG3, (D) IgM.

6.3.3 The IgG1 response to AMA1 is predominant over the study period

Standardised measures of antibodies were plotted over the entire period for each child present at all sample times as described in chapter 5 (Figure 6.4). Similar to the antibody plots for total IgG, antibody profiles of IgG1 could be described in three groupings. A number of children maintained a high antibody response throughout the study period whereas a smaller group had highly fluctuating antibody responses. The third group was made up of a considerable number of children who maintained a low antibody response (below 0.5 OD units) over the entire study period (Figure

6.4A-B). Most IgG3 responses were maintained at low levels throughout the study period with a few individuals exhibiting minor fluctuations and only two individuals maintaining high antibody levels throughout the study period (Figure 6.4C). All children had low levels of anti-AMA1(3D7) IgM throughout the study with a single child exhibiting a single peak in October 2003 (Figure 6.4D).

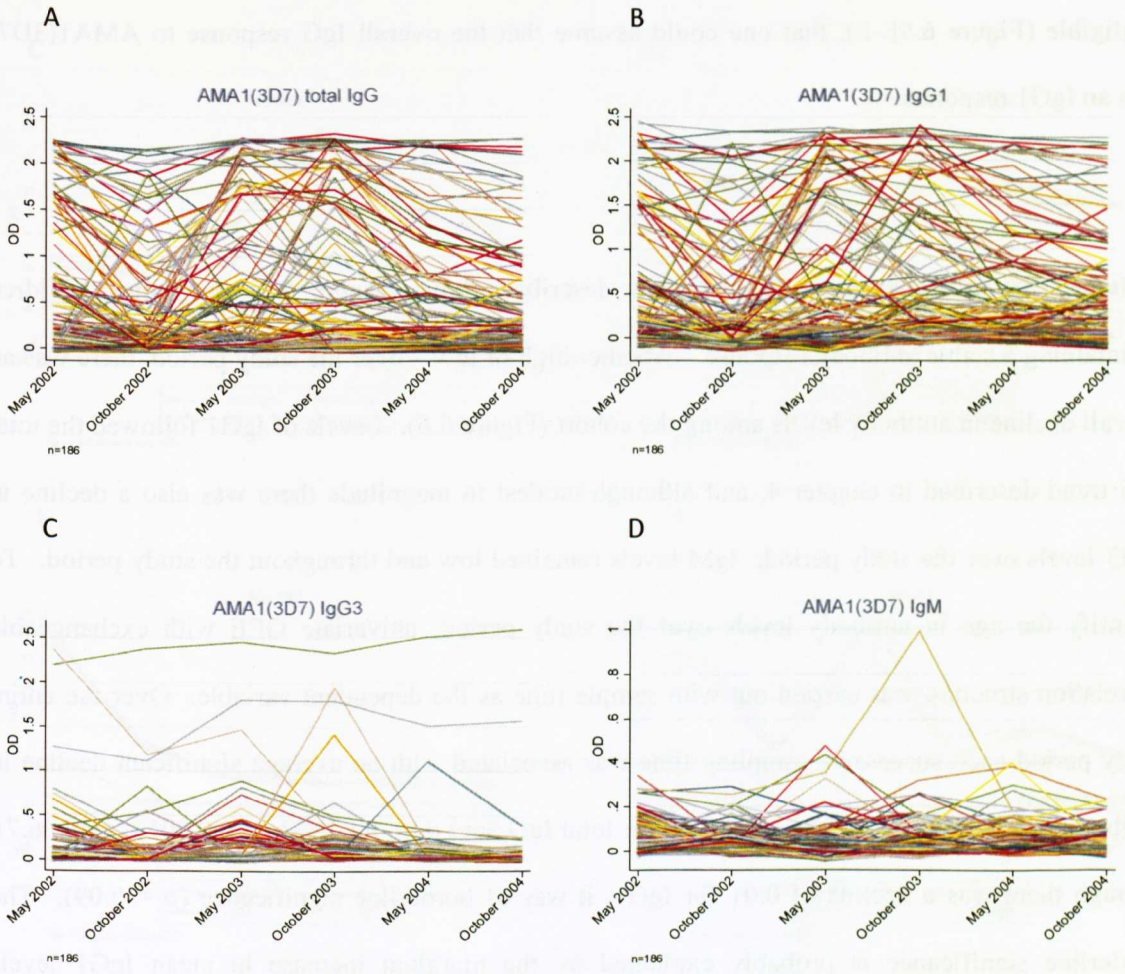

Figure 6.4 Longitudinal antibody responses to *P. falciparum* AMA1(3D7) for children present at all six sample times. Each line represents an individual. (A) Total IgG, (B) IgG1, (C) IgG3, (D) IgM.

Longitudinal antibody profiles of selected children who were below 2 years at the start of the study were similar with regards to all IgG and IgM responses (Figures 6.5A-B). Only in a few individuals could a predominant IgG1 response that mirrored the total IgG response be observed. In those children who were aged 2 or above at the start of the study, a predominantly IgG1 response became more common although a number of children still exhibited isotype and subclass responses that had similar patterns (Figure 6.5C-D). In the oldest age group, most children had high IgG1 responses that were so similar to the total IgG response and IgG3 responses that were negligible (Figure 6.5E-F), that one could assume that the overall IgG response to AMA1(3D7) was an IgG1 response.

Although the examples of individual plots described above (Figure 6.5) show most children maintaining a stable antibody response – whether high or low – over the study period, there was an overall decline in antibody levels among the cohort (Figure 6.6). Levels of IgG1 followed the total IgG trend described in chapter 4, and although modest in magnitude there was also a decline in IgG3 levels over the study period. IgM levels remained low and throughout the study period. To quantify the age in antibody levels over the study period, univariate GEE with exchangeable correlation structure was carried out with sample time as the dependent variable. Over the entire study period each successive sampling time was associated with an average significant decline in antibody levels with β coefficients of 0.02 for total IgG, and 0.01 for IgG3 ($p < 0.001$) (Table 6.7). Though there was a decline of 0.01 for IgG1, it was of borderline significance ($p = 0.09$). The borderline significance is probably explained by the marginal increase in mean IgG1 levels between May 2002 and October 2003 which then dropped at the same rate as the total IgG responses to levels below those seen at the start of the study.

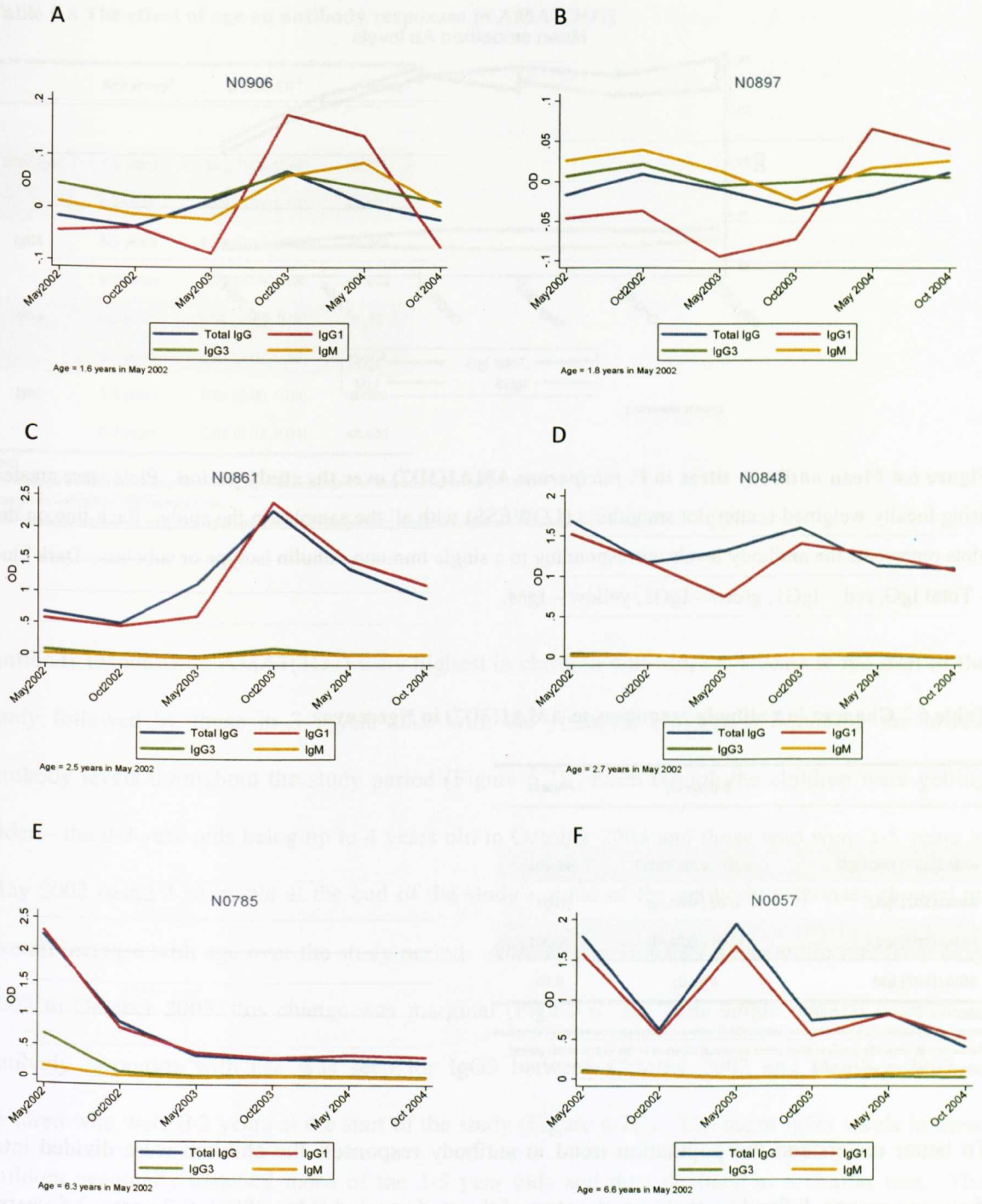

Figure 6.5 Longitudinal antibody (IgG) responses to *P. falciparum* AMA1(3D7) for selected individuals of different ages at the start of the study; 0-2 years (A and B), 3-5 years (C and D), 6-7 years (E and F). Eacch graph represents a single individual. Each line represents the antibody levels corresponding to a single immunoglobulin isotype or subclass. Dark blue – Total IgG, red – IgG1, green – IgG3, yellow – IgM.

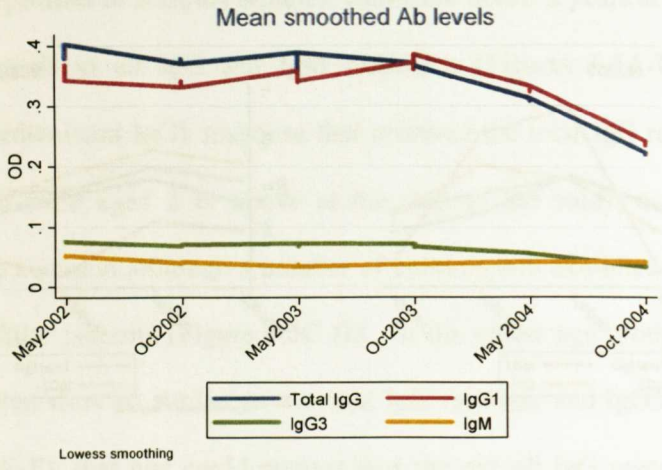

Figure 6.6 Mean antibody titres to *P. falciparum* AMA1(3D7) over the study period. Plots were created using locally weighted scatterplot smoothing (LOWESS) with all the samples in the study. Each line on the plots represents the antibody levels corresponding to a single immunoglobulin isotype or subclass. Dark blue – Total IgG, red – IgG1, green – IgG3, yellow – IgM.

Table 6.7 Changes in antibody responses to AMA1(3D7) in Ngerenya

	β (95% CI)[‡]	*P*-value
AMA1(3D7) Total IgG	-0.02 (-0.03, -0.01)	**<0.001**
AMA1(3D7) IgG1	-0.01 (-0.01, 0)	0.09
AMA1(3D7) IgG3	-0.01 (-0.01, 0)	**<0.001**
AMA1(3D7) IgM	0 (0, 0)	0.56

Notes
Univariate generalized estimating equation (GEE) with exchangeable correlation structure.
[‡]Reduction in antibody levels at each cross sectional bleed over the entire study period.
P-values ≤0.05 indicated in bold.

To better understand the population trend in antibody responses, the children were divided into three age groups defined by their age the start of the study period (May 2002); 0-2 years, 3-5 years, and 6-7 years. Mean antibody levels were plotted for each of the three age categories using Lowess smoothing techniques and differences in antibody levels throughout the study period were analysed by GEE (Table 6.8).

Table 6.8 The effect of age on antibody responses to AMA1(3D7)

	Age group[Δ]	β (95% CI)[†]	*P*-value
Total IgG	3-5 years	0.23 (0.10, 0.36)	**0.001**
	6-7 years	0.68 (0.52, 0.84)	**<0.001**
IgG1	3-5 years	0.24 (0.11, 0.37)	**<0.001**
	6-7 years	0.72 (0.56, 0.88)	**<0.001**
IgG3	3-5 years	0.02 (-0.03, 0.08)	0.40
	6-7 years	0.09 (0.03, 0.16)	**0.004**
IgM	3-5 years	0.01 (0.00, 0.03)	**0.008**
	6-7 years	0.03 (0.02, 0.04)	**<0.001**

Notes

Univariate generalized estimating equation (GEE) with exchangeable correlation structure (n=299).

[Δ] Age group during May 2002 sample time.

[†]GEE regression coefficients indicating reduction/increase in the antibody response compared to children age 0-2 years.

Antibody responses to AMA1(3D7) were highest in children who were 6-7 years at the start of the study followed by those in 3-5 year olds with the youngest children maintaining the lowest antibody levels throughout the study period (Figure 6.7). Even though the children were getting older – the 0-2 year olds being up to 4 years old in October 2004 and those who were 3-5 years in May 2002 being 7 years old at the end of the study – none of the antibody responses showed an overall increase with age over the study period. Although IgG1 levels appeared to rise from May 2002 to October 2003, this change was marginal (Figure 6.7B). The single marked increase in antibody responses with age was seen for IgG3 between October 2002 and October 2003 in children who were 0-2 years at the start of the study (Figure 6.7C). The mean IgG3 levels in these children eventually matched those of the 3-5 year olds and then declined at a similar rate. The increase in antibody levels from one age grouping to the next was significant for all antibodies (GEE: p <0.01). Antibody levels in the two older age groups compared with the youngest children were significantly higher (GEE: p <0.01) with mean antibody levels increasing up to 0.72 units (95% CI: 0.56, 0.88) between 0-2 year olds and 6-7 year olds for IgG1 (Table 6.8). As indicated by the IgG3 plot, there was a slight non-significant difference in the overall antibody response of 3-5 year olds compared to 0-2 year olds of 0.02 OD units (95% CI: -0.03, 0.08; p = 0.40).

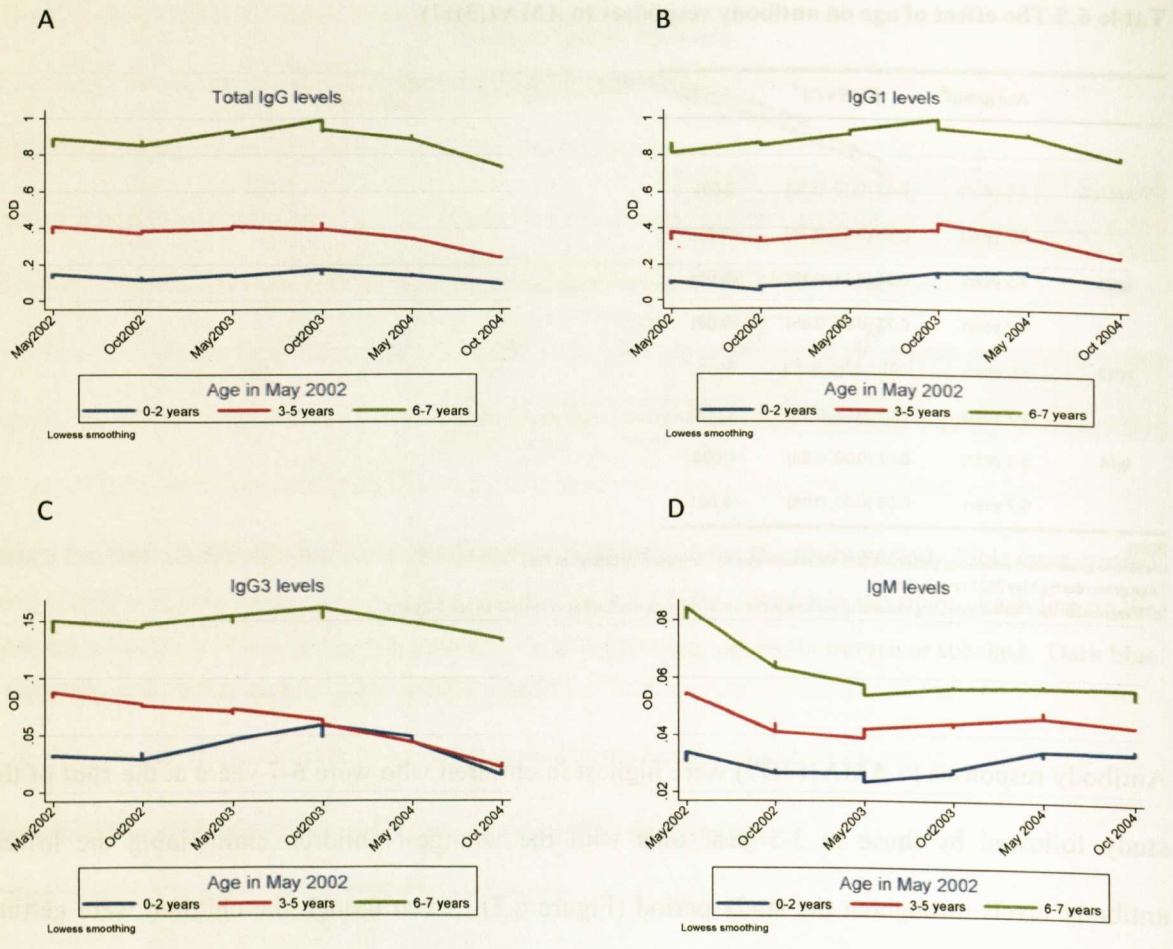

Figure 6.7 Mean antibody (IgG) titres to AMA1(3D7) over the study period in different age groups. Plots were created using locally weighted scatter plot smoothing (LOWESS) with all the samples in the study. Each line on the plots represents the antibody levels corresponding to an age grouping at the start of the study; 0-2 years (blue), 3-5 years (red), 6-7 years (green). (A) Total IgG (B) IgG1, (C) IgG3, (D) IgM.

6.3.4 Increasing episodes of clinical malaria do not affect the balance of IgG1 and IgG3 responses to AMA1

To investigate the hypothesis that repeated episodes of malaria leads to a polarisation of the IgG response from a mixed subclass response to a predominantly IgG1 response, mean antibody titres were plotted for children who had no exposure, those who had no clinical episodes, those who had one clinical episode and those who had two or more clinical episodes during the study (Figure 6.8). In all instances IgG1 was the predominant response. IgG3 responses remained very low even when children had been exposed though did not have any clinical malaria (Figure 6.8B). Those with one or more clinical episodes during the study had increasing IgG1 responses over time with no

significant change in the IgG3 response (Figure 6.8C-D); this further indicates that exposure to malaria is mainly inducing IgG1 to AMA1. In the last 12 months of the study there were no recorded episodes of clinical malaria and very little parasitaemia at cross sectional bleeds which probably explains why all antibody levels declined over that time.

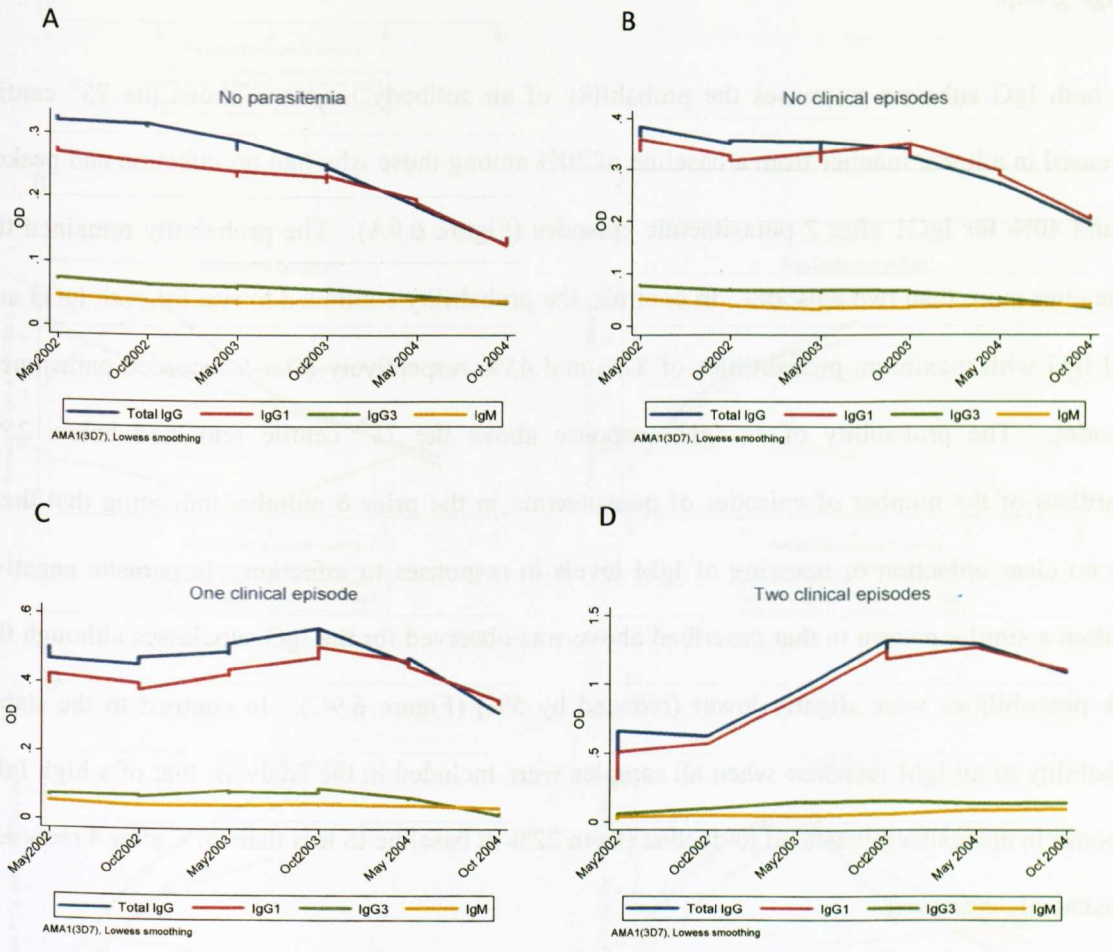

Figure 6.8 Mean antibody titres to *P. falciparum* AMA1(3D7) over the study period in children with different outcomes. (A) No parasitaemia, (B) no clinical episodes, (C) one clinical episode, and (D) two or more clinical episodes during the study period. Each line on the plots represents the mean antibody level corresponding to a single immunoglobulin isotype or subclass. Dark blue – Total IgG, red – IgG1, green – IgG3, yellow – IgM.

6.3.5 Effect of *P. falciparum* infection on boosting and induction of antibodies to AMA1

Assessment of the likelihood that recorded episode of *P. falciparum* infection in the six months prior to sampling results in high antibody titres was carried out by logistic regression as detailed in chapter 3. The probability of a positive antibody response above the 75th centile was calculated for all samples, and stratified by the presence or absence of concurrent parasitaemia at sampling and by age group.

For both IgG subclass responses the probability of an antibody response above the 75th centile increased in a linear manner from a baseline of 20% among those who had no infection and peaked around 40% for IgG1 after 2 parasitaemic episodes (Figure 6.9A). The probability remained the same after more than two episodes. In contrast, the probability continued to rise for both IgG3 and total IgG with maximum probabilities of 52% and 45% respectively after 5 recorded parasitmeic episodes. The probability of an IgM response above the 75th centile remained below 25% regardless of the number of episodes of parasitaemia in the prior 6 months, indicating that there was no clear induction or boosting of IgM levels in responses to infection. In parasite negative children a similar pattern to that described above was observed for the IgG subclasses although the peak probabilities were slightly lower (reduced by 5%) (Figure 6.9C). In contrast to the stable probability of an IgM response when all samples were included in the analysis; that of a high IgM response in aparasitaemic tended to decline (from 22% at baseline to less than 20% after 4 recorded parasitaemic episodes).

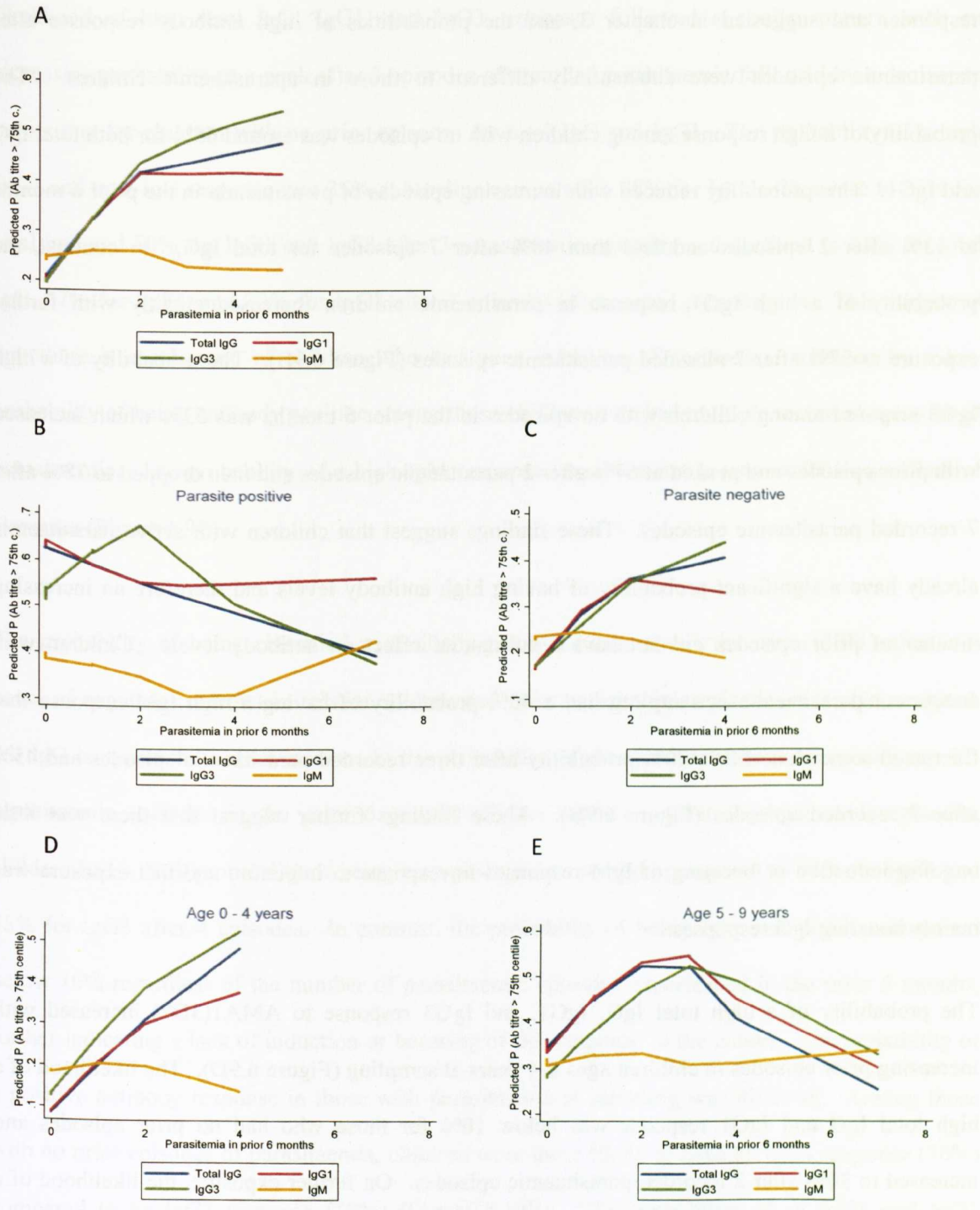

Figure 6.9 The predicted probability of antibodyresponses to AMA1(3D7) above the 75th centile by the number of recorded parasitaemic episodes in the 6 months prior to sampling. Each antibody response is represented by a single line on each graph; dark blue = Total IgG, maroon = IgG1, green = IgG3, yellow = IgM. (A) Predicted probability of antibody titres above the 75th centile in all samples, (B) parasite positive at sample time, and (C) parasite positive at sample time. Predicted probability of antibody titres above the 75th centile in children ages 0-4 years (D) and ages 5-9 years (E) at sampling.

Children with concurrent parasitaemia at sampling had a higher probability of being a high responder and suggested in chapter 3, and the probabilities of high antibody responses after parasitaemia episodes were substantially different to those in aparasitaemic children. The probability of a high response among children with no episodes was around 65% for both total IgG and IgG1. This probability reduced with increasing episodes of parasitaemia in the prior 6 months to 53% after 2 episodes and less than 40% after 7 episodes for total IgG. In contrast, the probability of a high IgG1 response in parasitaemic children increased slightly with further exposure to 57% after 7 recorded parasitaemic episodes (Figure 6.9B). The probability of a high IgG3 response among children with no episodes in the prior 6 months was 53% which increased with prior episodes and peaked at 67% after 2 parasitaemic episodes and then dropped to 38% after 7 recorded parasitaemic episodes. These findings suggest that children with active parasitaemia already have a significant probability of having high antibody levels and therefore an increasing number of prior episodes did not have a substantial effect on antibody levels. Children with concurrent parasitaemia at sampling had a 40% probability of having a high IgM response that fluctuated somewhat with a 30% probability after three recorded parasitaemic episodes and 45% after 7 recorded episodes (Figure 6.9B). These findings further suggest that there was little ongoing induction or boosting of IgM responses in response to infection, and that exposure was mainly boosting IgG responses.

The probability of a high total IgG, IgG1, and IgG3 response to AMA1(3D7) increased with increasing prior episodes in children ages 0-4 years at sampling (Figure 6.9D). The likelihood of a high total IgG and IgG1 response was below 10% for those who had no prior episodes and increased to 30% after 2 recorded parasitaemic episodes. On further exposure, the likelihood of a high IgG1 response did not increase as much and peaked at 35% for IgG1 and 45% for total IgG after four episodes. Although the predominant response to AMA1 was IgG1, the likelihood of a high IgG3 response was always greater than that of IgG1. Without any prior exposure in the past 6 months, a child aged 0-4 years had a 14% probability of a high IgG3 response that rose with increasing exposure to 52% after 4 recorded parasitaemic episodes (Figure 6.9D). In contract to the IgG responses, the probability of a high IgM response fell from 20% at baseline to 13% after 4 recorded parasitaemic episodes. Older children (ages 5-9 years) were more likely to have high

antibody responses even in the absence of prior exposure (Figure 6.9E). Among this age group, the likelihood of high total IgG, IgG1, and IgG3 responses followed a similar pattern with an increasing probability to a peak after 3 episodes of recorded parasitaemia followed by a decline to below that for children with no prior episodes for total IgG and IgG1. The probability of a high IgG1 and total IgG response was 35% at baseline compared to 28% for IgG3. This rose to 55% for total IgG and 50% for IgG1 and IgG3 after 3 parasitaemic episodes and declined to 25% for total IgG, 27% for IgG1, and 30% for IgG3 after 7 episodes of recorded parasitaemia. This suggested that there is a subgroup of children who had poor induction of antibodies, reflected by a low probability of being antibody positive among those children who had multiple recorded episodes of parasitaemia. The probability of a high IgM response remained stable around 30% regardless of exposure (Figure 6.9E).

The probability of being classified as antibody positive (titres above the mean+3SD value of a panel of negative controls) in relation to recorded prior parasitaemia episodes was also analysed. All IgG isotypes showed an increase in the probability of sero-positivity from no episodes to 2-3 parasitaemic episodes in the prior 6 months (Figure 6.10A). After 3 parasitaemic episodes, children had a 70% probability of being sero-positve for total IgG compared to 55% for IgG1 and 35% for IgG3 after 4 episodes. In contrast, the probability of being IgM sero-positive remained below 10% regardless of the number of parasitaemic episodes experienced in the prior 6 months, further indicating a lack of induction or boosting of this response in the cohort. The probability of a positive antibody response in those with parasitaemia at sampling was different. Among those with no prior episodes of parasitaemia, children were more likely to have an IgG1 response (70%) compared to an IgG3 response (50%) (Figure 6.10B). The probability of an IgG1 and IgG3 response increased with increasing exposure and peaked at 60% after 2 episodes of recorded parasitaemia for IgG3 compared with a decline to 60% after 2 episodes for IgG1. Further exposure increased the probability of an IgG1 response to 80% after 7 episodes of recorded parasitaemia. In contrast, the probability of an IgG3 response declined to 40% after 7 parasitaemic episodes. Further exposure did not increase the likelihood of an IgG response. The probability of an IgM response to AMA1(3D7) remained below 20% regardless of concurrent parasitaemia or increasing

prior exposure, though there was a steady decline in the likelihood with increasing exposure in parasitaemic children (Figure 6.10B). These findings again suggest that most children with active parasitaemia already have antibodies and therefore an increasing number of prior episodes does not have a substantial effect on antibodies.

When stratified by age, the probability of all the tested antibody responses to AMA1(3D7) were similar in children ages 0-4 years as those described above when all the samples were analysed together although the peak probabilities never rose above 65% (Figure 6.10D). This was not the case for older children. Among children aged 5-9 years, the probability of being antibody positive was 50%, 38%, and 20% for total IgG, IgG1 and IgG3 responses respectively, for those with no prior episodes (Figure 6.10E). The probability of these responses increased at a similar rate to 80%, 60%, and 40% respectively after 3 parasitaemic episodes and declined to 50% for total IgG and IgG1 and 20% for IgG3 after 7 episodes. The likelihood of a detectable IgM response in older children remained around 10% regardless of the amount of prior exposure (Figure 6.10E).

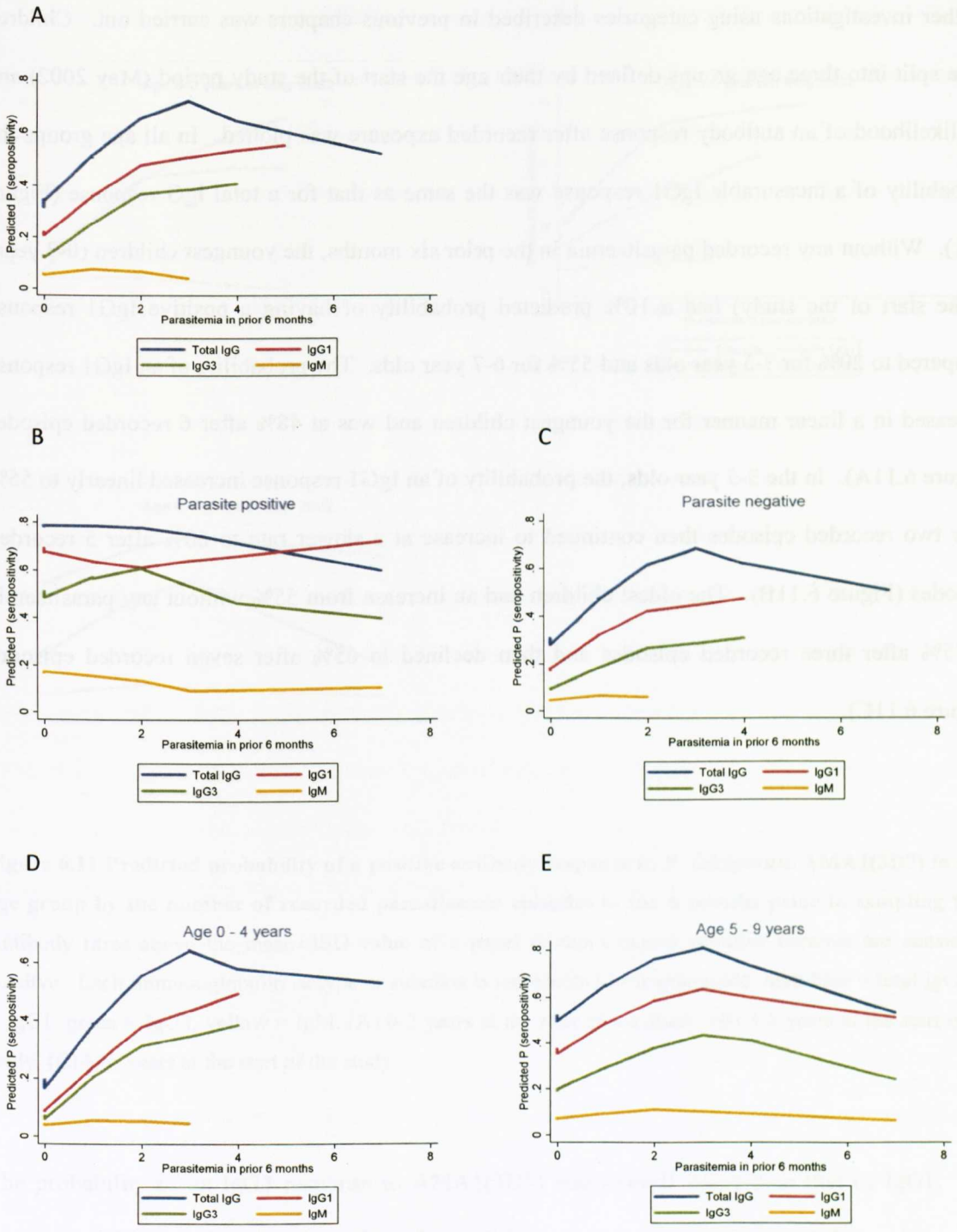

Figure 6.10 The predicted probability of detectable antibody responses to AMA1(3D7) by the number of recorded parasitaemic episodes in the 6 months prior to sampling. Each antibody response is represented by a single line on each graph; dark blue = Total IgG, maroon = IgG1, green = IgG3, yellow = IgM. (A) Predicted probability of sero-positive responses in all samples, (B) parasite positive at sample time, and (C) parasite positive at sample time. Predicted probability of sero-positive responses in children ages 0-4 years (D) and ages 5-9 years (E) at sampling.

Further investigations using categories described in previous chapters was carried out. Children were split into three age groups defined by their age the start of the study period (May 2002) and the likelihood of an antibody response after recorded exposure was plotted. In all age groups the probability of a measurable IgG1 response was the same as that for a total IgG response (Figure 6.11). Without any recorded parasitaemia in the prior six months, the youngest children (0-2 years at the start of the study) had a 10% predicted probability of having a positive IgG1 response compared to 20% for 3-5 year olds and 55% for 6-7 year olds. The probability of an IgG1 response increased in a linear manner for the youngest children and was at 48% after 6 recorded episodes (Figure 6.11A). In the 3-5 year olds, the probability of an IgG1 response increased linearly to 55% after two recorded episodes then continued to increase at a slower rate to 60% after 5 recorded episodes (Figure 6.11B). The oldest children had an increase from 55% without any parasitaemia to 75% after three recorded episodes and then declined to 65% after seven recorded episodes (Figure 6.11C).

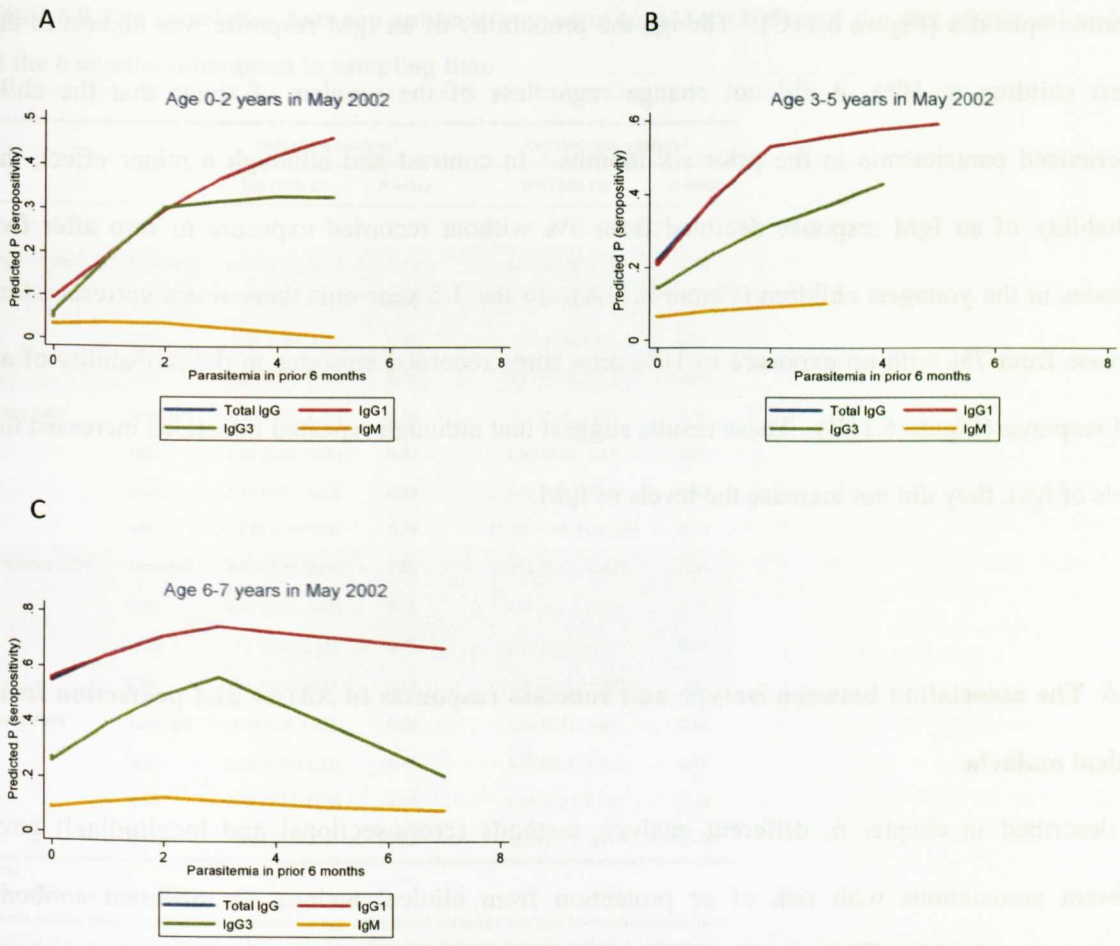

Figure 6.11 Predicted probability of a positive antibody response to *P. falciparum* AMA1(3D7) in each age group by the number of recorded parasitaemic episodes in the 6 months prior to sampling time. Antibody titres above the mean+3SD value of a panel of non-exposed negative controls are considered poisitve. Each immunoglobulin isotype or subclass is represented by a single line; dark blue = total IgG, red = IgG1, green = IgG3, yellow = IgM. (A) 0-2 years at the satrt of the study, (B) 3-5 years at the start of the study, (C) 6-7 years at the start of the study.

The probability of an IgG3 response to AMA1(3D7) was overall lower than that of IgG1. The youngest children had a 5% probability of an IgG3 response without any recorded parasitaemia in the prior six months compared to 15% for the 3-5 year olds and 25% for the oldest children. The probability of an IgG3 response increased at the same rate as that of an IgG1 response in the youngest children but peaked and remained at 30% after two recorded episodes (Figure 6.11A). In 3-5 year old children, the probability of an IgG3 response increased linearly to 40% after 4 episodes. Like the IgG1 response in the youngest children, the probability of an IgG3 response in 3-5 year olds continued to rise with no changes (Figure 6.11B). After three recorded episodes, the probability of an IgG3 response peaked at 50% in the oldest children and fell to 20% after seven

recorded episodes (Figure 6.11C). Though the probability of an IgM response was highest in the oldest children at 10%, it did not change regardless of the number of times that the child experienced parasitaemia in the prior six months. In contrast and although a minor effect, the probability of an IgM response declined from 5% without recorded exposure to zero after five episodes in the youngest children (Figure 6.11A). In the 3-5 year olds there was a corresponding increase from 7% with no exposure to 10% after three recorded episodes in the probability of an IgM response (Figure 6.11B). These results suggest that although repeated infections increased the levels of IgG, they did not increase the levels of IgM.

6.3.6 The association between isotype and subclass responses to AMA1 and protection from clinical malaria

As described in chapter 6, different analysis methods (cross-sectional and longitudinal) gave different associations with risk of or protection from clinical malaria for different antibody responses when a single sample point was used in the cross sectional analysis. This was also the case for IgG1, IgG3, and IgM responses. All three antibody responses were associated with an increased risk of disease in the subsequent six months when analysed cross-sectionally in May 2002 (Table 6.9). The only association that approached significance was that with the IgG1 response (IRR 1.52, 95% CI 0.97 - 2.38, p = 0.07). There were no statistically significant associations for IgM (Eg. IRR 0.05, 95% CI 0.00 - 5.98, p = 0.22 for May 2003). When all the samples were analysed using a clustering indicator, IgG1 and IgG3 responses had no association with the risk of clinical malaria in the six months subsequent to sampling in both the univariate and multivariable analysis. Though IgM responses appeared to be associated with protection from malaria in both the univariate and multivariable analysis (IRR 0.27 and 0.53), none of these effects were statistically significant (Table 6.9).

Table 6.9 The association between antibody responses to AMA1(3D7) and the risk of clinical malaria in the 6 months subsequent to sampling time

		Univariate analysis[‡]		Multivariable analysis[◊]	
		IRR (95% CI)	*P*-value	IRR (95% CI)	*P*-value
All samples[Δ]	Total IgG	1.09 (0.87, 1.38)	0.46	1.02 (0.78, 1.33)	0.88
	IgG1	1.09 (0.85, 1.41)	0.50	1.03 (0.77, 1.38)	0.84
	IgG3	1.05 (0.64, 1.7)	0.86	1.05 (0.6, 1.82)	0.87
	IgM	0.27 (0.01, 5.79)	0.40	0.53 (0.03, 8.97)	0.66
May 2002[†]	Total IgG	1.36 (0.85, 2.16)	0.20	1.2 (0.71, 2.05)	0.50
	IgG1	1.52 (0.97, 2.38)	**0.07**	1.39 (0.83, 2.32)	0.21
	IgG3	1.56 (0.57, 4.25)	0.39	1.46 (0.47, 4.61)	0.51
	IgM	17 (0.1, 2839.3)	0.28	13.89 (0.06, 3182.87)	0.34
October 2002[†]	Total IgG	1.03 (0.79, 1.36)	0.81	0.91 (0.67, 1.23)	0.54
	IgG1	1.07 (0.82, 1.41)	0.61	0.95 (0.71, 1.28)	0.74
	IgG3	0.8 (0.24, 2.66)	0.72	0.74 (0.21, 2.59)	0.64
	IgM	0.95 (0.05, 16.59)	0.97	0.85 (0.04, 16.97)	0.91
May 2003[†]	Total IgG	1.09 (0.8, 1.47)	0.59	1.02 (0.72, 1.44)	0.92
	IgG1	1.12 (0.83, 1.51)	0.45	1.07 (0.76, 1.51)	0.71
	IgG3	1.43 (0.54, 3.79)	0.48	1.64 (0.53, 5.08)	0.40
	IgM	0.05 (0, 5.98)	0.22	0.14 (0, 15.95)	0.42

Notes

[‡] Univariaable poisson regression.

[◊] Multivariable poisson regression (adjusted for age, concurrent parasitaemia, and haemoglobinopathies)

[Δ] Analysis of all samples (all 6 sampling points) with the addition of an indicator for those children contributing more than one observation to the analysis.

[†] Analysis covering the 6 month period subsequent to the cross sectional bleed indicated.

No analysis for October 2003, May 2004, and October 2004 cross-section bleeds due to lack of malaria episodes after the October 2003 sampling.

P-values ≤0.1 indicated in bold.

Multivariable GEE was carried out to investigate the overall effect on antibody levels of all the factors discussed in chapter 3. Both recurrent parasitaemia at sample time and being in the highest age category (6-7 years at the start of the study) significantly increased antibody levels to the same degree (β=0.57, p<0.001). Though prior parasitaemia also had a significant association (p<0.001) with total IgG levels, it was not as high as of age and recurrent parasitaemia. In the case of IgG1, age (6-7 years at the start of the study) and recurrent parasitaemia significantly increased antibody levels by the greatest degree. Contrasting with the total IgG and IgG1 results, age was not associated with IgG3 responses to the same degree (β=0.07, p=0.02). Recurrent parasitaemia was the strongest predictor of IgG3 responses (β=0.17, p<0.001). Though significant, the effects of age and recurrent parasitaemia on IgM levels were much smaller than those on IgG subclasses (β=0.02 and β=0.03 respectively, both p<0.001). Though the haemoglobinopathies led to a reduction in all antibody responses, the only significant effect was that of the sickle trait on total IgG levels (p=0.03).

Longitudinal analysis of the association between antibody responses and protection from malaria gave slightly different results to those seen in the cross-sectional analysis with clustering (Table 6.10). In the multivariable GEE, IgG1 and IgG3 responses were not associated with a higher or lower risk of malaria over the entire study period. IgM responses were associated with protection in both the univariate and multivariable analysis (IRR 0.10 and 0.27) though they were not statistically significant. Even after removal of those children who had not been exposed over the study period the relationships remained the same; IgG1, IgG3, and IgM had no significant association with the risk of malaria (Table 6.10).

Table 6.10 The effect of antibody responses to AMA1(3D7) on the risk of malaria over the study period.

	Univariate analysis[°]		All children[Δ]		Exposed children[†]	
	IRR (95% CI)	*P*-value	IRR (95% CI)	*P*-value	IRR (95% CI)	*P*-value
Total IgG	1.07 (0.88, 1.31)	0.50	1.04 (0.82, 1.31)	0.75	1.01 (0.81, 1.25)	0.94
IgG1	1.04 (0.84, 1.28)	0.72	1.02 (0.8, 1.29)	0.90	0.98 (0.78, 1.22)	0.84
IgG3	0.94 (0.46, 1.89)	0.85	0.92 (0.43, 1.97)	0.83	0.89 (0.43, 1.8)	0.74
IgM	0.1 (0.01, 1.44)	**0.09**	0.27 (0.02, 3.37)	0.31	0.18 (0.02, 2.19)	0.18

Notes
[°] Univariate generalised estimating equation (GEE).
[Δ] Multivariable GEE, exchangeable correlation structure. All samples.
[†] Multivariable GEE, exchangeable correlation structure. Only those children with recorded *P. falciparum* exposure during the study.
P-values ≤0.1 indicated in bold.

6.4 Discussion

In agreement with published findings (Riley et al. 2000; Polley et al. 2004; Metenou et al. 2007; Nebie et al. 2008; Stanisic et al. 2009), IgG1 was the predominant subclass response to AMA1(3D7) from the age of 3 years. This IgG1 predominance was also seen over the entire duration of the study. Increasing age led to higher IgG1 responses at all cross sectional bleeds which is in agreement with recent findings in Papua New Guinea (Stanisic et al. 2009). Unlike the study mentioned, IgG3 levels significantly increased in one year intervals rather than every six months like IgG1. This result points to a slower acquisition of IgG3 responses to AMA1. In agreement with Stanisic *et al* there was no evidence that increasing age led to polarisation of the

antibody response to AMA1 as was seen in previous studies (Taylor et al. 1998; Tongren et al. 2006). The longitudinal nature of this study made it particularly good at examining whether polarisation of IgG subclass responses occurs over time. Although it may be argued that the reduction in malaria transmission during the course of this study would make it difficult to address whether polarisation actually occurred, the weekly follow-up allowed us to indentify children with varying amounts of exposure and plot their IgG1 and IgG3 responses over time.

The acquisition and maintenance of the IgG1 response was very similar to that of the overall IgG response, whereas that of IgG3 differed somewhat. One or two recorded episodes of parasitaemia in the 6 months prior to sampling time was associated with an increased probability of having high IgG, IgG1, and IgG3 levels. More than three prior parasitaemic episodes were associated with a lower probability of a high titre IgG1 response. This result appears to indicate that there is a cap on the probability of a high titre response. In children who are still in the active stages of acquiring malaria immunity, the propensity to mount an IgG1 response was similar to that of IgG3 up to two prior episodes. Further episodes result in a predominantly IgG1 response. This could be due to the relative immaturity of the immune cells which leads to an initial mixed IgG subclass response. On further exposure the nature of the antigen may have a stronger effect thus leading to a predominantly IgG1 response. Above the age of two years children may have accumulated sufficient exposure to the antigen that would lead to a predominantly IgG1 response. Both these points are supported by the fact that the strongest factor associated with antibody levels was recurrent exposure.

IgG3 was the minor subclass response to AMA1(3D7). Unlike the dominant IgG1 subclass, IgG3 was titres were not reduced in children with haemoglobinopathies. In agreement with published data, the overall levels remained low throughout the study period. The slight increase in IgG3 levels seen between October 2002 and October 2003 in the youngest children (0-2 years at the start of the study) could be attributed to the initial stages of exposure in relatively naive immune systems which result in a mixed response dominated by IgG1 but where IgG3 is also present. This is supported by the fact that prevalence of IgG1 and IgG3 was similar in children until the age of

three when IgG1 began to dominate. Therefore it is possible that the highest IgG antibody levels would be reached after few exposures and any further exposure would not lead to increased levels.

IgM is induced on primary exposure to antigen therefore low prevalence is expected in areas where malaria is endemic, as was the case in this study. The fact that IgG and IgM responses to AMA1 do not strongly correlate with each other is in agreement with the idea that increasing malaria exposure boosts secondary responses (IgG) and induces a memory response. A high correlation might suggest that this memory response is not being generated effectively. As the children in this study were continuously being exposed to Plasmodium, it was unlikely that we were observing primary exposure except in the youngest children. This would explain the low IgM levels throughout the study. The higher levels seen in older children were still relatively low when compared to the IgG response. Because IgM is induced on initial exposure, we did not expect any further exposure of the same antigen, whether or not it results in clinical disease to induce an IgM response. This result was in agreement with the concepts of IgM induction on primary exposure and not evidence of a failure in isotype switching as has been suggested (Scopel et al. 2006; Tongren et al. 2006). As the disease-causing parasite and its corresponding antigen are more likely than not to be of a heterologous strain, measurement of the IgM response to one variant – AMA1(3D7) in this case – would be unable to capture any induction in IgM that may result from exposure to new variants. The probability of an IgM response considered sero-positive remained low regardless parasitaemia episodes. The low IgM titres observed were due to the fact that most of the children were already exposed at the start of the study and this continued throughout the study even as transmission dropped. Any IgM peaks could not be detected due to the wide sampling times. The relatively stable probability of a high titre IgM response on increasing exposure may indicate that on every exposure to any antigen, regardless of primary or secondary exposure, there is a certain amount of IgM that is produced by the immune system.

Total IgG responses to AMA1(3D7) did not have any effect on protection from clinical malaria in previous chapters so it is not surprising that the IgG1 response also had no association with protection as this was the predominant IgG subclass response. This resulted contrasted with that found in Burkina Faso where IgG1 was associated with protection from clinical malaria (Nebie et

al. 2008). The lack of association for IgG1 with protection has also been suggested in another study (Stanisic et al. 2009). Unlike the Stanisic *et al* study IgG3 was not associated with protection from malaria. The protection seen with regards to IgG3 in that study is surprising as one would not expect the minor subclass response to be that which will confer protection. It has been suggested that differences in population genetics, age groups, and malaria transmission could be the reason for this result.

Increasing IgG1 titres in association with increasing exposure and disease suggest that this response to AMA1(3D7) could be a marker of exposure rather than one of protection in this cohort. Although statistically non-significant, only IgM responses were associated with protection from clinical disease. If the low affinity nature of IgM allows it to bind to different variants of the same antigen then this association with protection could be due to the IgM interacting with another AMA1 variant on the disease causing parasite. This non-specific response along with other more specific IgG responses could end up protecting the individual from disease. Due to time constraints investigation of the subclass and isotype response in another antigen was not possible. Future work is planned for MSP2(3D7) as an example of an antigen that induces an IgG3 dominant response.

7 Acquisition and prevalence of antibodies to specific epitopes of AMA1 and their association with invasion inhibition and protection from clinical malaria

7.1 Introduction

Passive transfer of antibodies from immune individuals was first shown to confer protection against malaria in the 1960s (Cohen et al. 1961). There are numerous *Plasmodium spp* proteins to which naturally acquired antibodies have been measured including the blood-stage antigens; apical membrane protein 1 (AMA1), merozoite surface proteins 1-3 (MSP1 – MSP3), and erythrocyte binding antigen 175 (EBA175) among others. Immuno-epidemiological studies also provide further evidence that these above mentioned antigens may be targets of protective immunity (Conway et al. 2000; Cavanagh et al. 2004; Polley et al. 2004).

Protection from disease in immunization-challenge studies with recombinant AMA1 in animal models has been observed (Deans et al. 1988; Collins et al. 1994; Crewther et al. 1996; Anders et al. 1998; Narum et al. 2000), although the AMA1 needs to be folded in its proper conformation in order to do so (Collins et al. 1994; Crewther et al. 1996; Anders et al. 1998). Antibodies to AMA1 are also detected in most members of malaria endemic populations (Thomas et al. 1994; Johnson et al. 2004; Polley et al. 2004; Chelimo et al. 2005; Cortes et al. 2005). Some immuno-epidemiological studies show that individuals with high levels of AMA1 specific antibodies are at a reduced risk of experiencing an episode of clinical malaria (Polley et al. 2004; Gray et al. 2007; Osier et al. 2008; Stanisic et al. 2009), although a couple have not indicated this protective effect of anti-AMA1 antibodies (Roussilhon et al. 2007; Nebie et al. 2008).

Antibodies to AMA1 are able to inhibit invasion of erythrocytes by merozoites in vitro (Hodder et al. 2001; Healer et al. 2004), and although most of inhibitory antibodies are targeted to

polymorphic regions of the protein, and inhibitory antibodies are generally strain specific, there is some evidence that invasion-inhibitory antibodies could also recognize conserved or less polymorphic epitopes (Kennedy et al. 2002; Collins et al. 2007). Inhibition of erythrocyte invasion by parasites appears to be variant specific as well as cross reactive. Antibodies produced by the immunisation of rabbits with recombinant 3D7 *Pf*AMA1 significantly inhibited invasion of *P. falciparum* field isolates and lab strains that share sequence similarity (eg. D10) whereas those antibodies generated by immunization with recombinant FVO *Pf*AMA1 were able to inhibit erythrocyte invasion by field isolates and well lab strains in with divergent AMA1 sequences (Duan et al. 2008). Though both animal and human antibodies to AMA1 can inhibit invasion of erythrocytes, most of the antibodies tested interact with strain-specific epitopes in order to exert this functionality (Hodder et al. 2001).

The crystal structure of AMA1 reveals a long hydrophobic region in domain I that is hypothesised to be a ligand-binding site. It is surrounded by a region consisting of the highest number of polymorphic residues in the protein (Bai et al. 2005). These polymorphisms appear to have arisen due to diversifying selection in order to avoid invasion-inhibitory antibodies (Cortes et al. 2003; Polley et al. 2003). Invasion inhibitory epitopes in domains II and III have also been described (Nair et al. 2002; Mueller et al. 2003; Collins et al. 2007). As a primary action of antibodies to AMA1 is inhibition of invasion, we set out to investigate the prevalence and acquisition of antibodies to specific polymorphic and invasion-inhibitory AMA1 epitopes in a malaria-endemic setting, and to relate these antibodies to protection from clinical malaria.

Monoclonal antibody 1F9 is strain specific and inhibits invasion of 3D7 and D10 *P. falciparum* parasites but not HB3 or W2mef, and recognises a polymorphic epitope in domain I (Coley et al. 2001; Coley et al. 2006). The D10 and 3D7 strains of AMA1 are identical in domain I but differ in domains II and III. The actual binding site of mAb 1F9 appears to be part of the hydrophobic trough and the surrounding loops on the AMA1 surface (Figure 7.1). This interaction between AMA1 and mAb 1F9 is quite large, conformationally dependent, and substitution of residue 197

completely inhibits the AMA1-1F9 interaction (Coley et al. 2006). The interface between AMA1 and 1F9 is made up of a large buried area that the antibody protrudes into. Though this interface is unusual, binding of mAb 1F9 does not significantly affect the structure of AMA1 (Coley et al. 2007). Antibodies from exposed individuals from Papua New Guinea compete with mAb 1F9 for binding to AMA1, whereas antibodies from non-malaria exposed individuals do not (Coley et al. 2007).

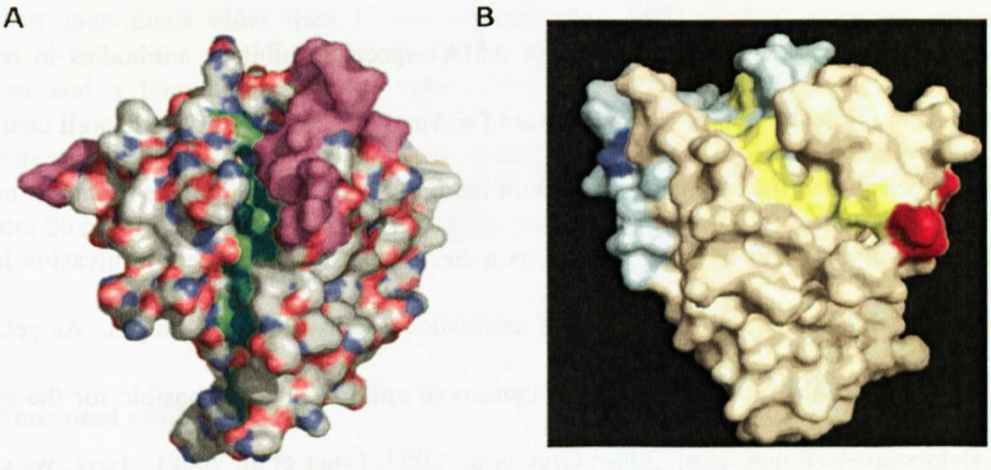

Figure 7.1 X-ray structure of *P. falciparum* AMA1 domains I and II showing the hydrophobic trough and binding sites of mAb 1F9 and mAb 4G2. (A) Base of the hydrophobic trough indicated in green, disordered loops in purple (Bai et al. 2005), (B) residue 197 that is critical for mAb 1F9 binding shown in blue, mAb 4G2 critical residues in red, and the hydrophobic cleft is in yellow (Coley et al. 2006).

Monoclonal antibody 2C5 also reacts with 3D7 and D10 *P. falciparum* AMA1. MAb 2C5 does not inhibit invasion of erythrocytes by merozoites (Coley et al. 2006). As the entire, correctly folded AMA1 ectodomain is necessary for reactivity, a distinct epitope separate from that of 1F9 but which is common to the 3D7 and D10 strains is suggested. MAb 5G8 is not strain specific in its interactions but only recognizes *P. falciparum* (Coley et al. 2006). It recognizes a 19 residue linear epitope in the N-terminal pro-sequence/pro-domain of AMA1 (Coley et al. 2001). As the prosequence is cleaved from the mature form of AMA1 expressed by *P. falciparum* merozoites we do not expect much malaria- and age-related reactivity to the mAb 5G8 epitope, but it will serve as a control.

Another invasion inhibitory monoclonal antibody that has been studied in detail is 4G2. Initial studies showed that 4G2 exerted its inhibitory effects not by simple steric effects but by binding to a functionally important part of AMA1. This same study revealed that the mAb 4G2 epitope was conserved across all *P. falciparum* variants and was situated in the domain II loop of AMA1 (Figure 1) (Coley et al. 2007). AMA1 forms a complex with the essential rhoptry neck protein RON4 which then associates with the moving junction during invasion. MAb 4G2 appears to exert its inhibitory effects by binding to the domain II loop before the AMA1/RON4 complex forms and therefore inhibits downstream invasion actions (Collins et al. 2009).

It has not been possible to directly measure AMA1-specifc inhibitory antibodies in relation to protection from clinical malaria. With the use of mAbs 1F9, 2C5 and 5G8 and well characterised cohorts it is possible to investigate the ability of naturally acquired antibodies to inhibit binding of monoclonal antibodies and use this activity as a surrogate of AMA1-specific invasion-inhibitory activity, and to understand the acquisition of antibodies to polymorphic epitopes. As yet, it is not clear whether antibodies to polymorphic or conserved epitopes are responsible for the protection seen in field studies (Polley et al. 2004; Gray et al. 2007; Osier et al. 2008). Here, we examined antibodies to two polymorphic epitopes (one inhibitory and another non-inhibitory) and their relationship with protection against clinical malaria.

7.2 Methods

7.2.1 Study populations, sample collection, and surveillance

Samples were obtained from cohort studies based in Kilifi District; a malaria-endemic area in Kenya. The study sites were two villages (Ngerenya and Chonyi) that have been part of a demographic surveillance system since the 1990s. The population is predominantly Mijikenda and rural. Most of the malaria in the area results from *P. falciparum* (Mwangi et al. 2003). Biannual malaria transmission occurs during the rainy seasons of May-July and November-December, with the shorter rains occurring later in the year. Ngerenya is considered a low-medium transmission area with an entomologic inoculation rate (EIR) of approximately 10 infective bites per person per

Mwangi 2003). Venous and/or finger prick blood samples and slide data have been collected from study participants every 6-12 months. The study samples comprised of 130 serum samples from Ngrerenya collected in September 1998 (ages 0-85 years) and 294 samples collected in October 2002 (ages 0-10 years). 276 samples from Chonyi were collected in October 2000 from individuals 0-55 years of age. All individuals were monitored weekly by active and passive surveillance for symptomatic illness and malaria; any febrile episodes were investigated for malaria. Malaria was defined as a temperature greater than 37.5°C and any parasitaemia in children less than 1 year of age. For individuals older than 1 year, malaria was defined as parasitaemia above 2500 parasites/μl and a temperature greater than 37.5°C. Age-specific malaria and parasitaemia incidence as well as prevalence rates have been determined in this population (Mwangi et al. 2005). Serum from 20 non-exposed British and Australian donors were used as reference controls.

7.2.2 Monoclonal antibodies

Monoclonal antibodies were obtained from Anders RF. They were generated using standard methods (Harlow and Lane 1988). MAb 1F9 and 5G8 were generated by immunization of mice with recombinant refolded 3D7 AMA1. MAb 1F9 was produced in hybridoma cell cultures and purified by protein G chromatography (Coley et al. 2007). The reactivity of the MAb to AMA1 was assayed by ELISA, and immunofluorescence of 3D7 schizonts and merozoites (Coley et al. 2001). The 4G2 mAb was not available to us for use in these studies.

7.2.3 Enzyme-linked immunosorbent assay (ELISA)

7.2.3.1 Standard ELISA

Total IgG to recombinant AMA1(3D7) and A4 schizont extract proteins was measured by standard ELISA methods. IgM to recombinant AMA1(3D7) was also measured using the same method. The protocol is described in chapter 2.

7.2.3.2 Competition ELISA

Antibodies to epitopes specific to three monoclonal antibodies (1F9, 2C5, and 5G8) on AMA1(3D7) were measured by a competitive ELISA method fully described in chapter 2.

7.2.4. Growth inhibition assay (GIA)

In vitro inhibition of *P. falciparum* growth by human serum was tested by GIA with the growth of parasites expressed as a percentage of the maximal growth achieved in control wells. Details of the methodology are described in chapter 2. The assays were performed by Fiona McCallum (McCallum et al. 2008).

7.2.5 Statistical analysis

Differences in median antibody levels were analysed using the Kruskal-Wallis test. Tests for trend were used to check for patterns in the proportion of the population acquiring a particular response. Antibody levels to recombinant AMA1 (3D7), the 1F9, 2C5, 5G8 epitopes, and parasite growth *in vitro* were compared using Spearman rank correlation. Kaplan-Meier survival functions were used to investigate the failure rates and characteristics of the groups described above. Differences in survival functions were tested using the Log-rank test. Differences in the likelihood of failure at any point during follow up between high and low responders was analysed using Cox Proportional Hazards. Both the unadjusted and age adjusted proportional hazards were investigated. The relationship between antibody levels and the risk of experiencing a clinical malaria episode in the 6 months post-sampling was analysed using univariate and then multivariate generalised linear models (GLM) that were adjusted for age (in 1 year categories) and previous *P. falciparum* exposure using reactivity to schizont extract (continuous variable). Risk was expressed as relative risk/risk ratio as the outcome incidence was above 10%; and was compared between those with high and low antibody responses (assigned using the median value) in the entire cohort as well as in groups stratified by parasite status. Significance was reached at a 0.05 level for all tests.

7.3 Results

7.3.1 Serological prevalence and reactivity to specific epitopes of AMA1 in malaria endemic settings

Samples from two areas representing low-medium (Ngerenya) and medium-high (Chonyi) malaria transmission settings were tested for antibodies that inhibit mAb 1F9, 2C5, and 5G8. 276 serum samples were collected in October 2000 from Chonyi (ages 0 – 55 years), 130 samples in September 1998 from Ngerenya (ages 2 – 81 years), and 294 samples in October 2002 also from Ngerenya (ages 0 – 8 years). Samples from both Ngrerenya and Chonyi were used in order to include adults for whom prior associations with protection for anti-AMA1 antibodies had been observed (Polley et al. 2004; Osier et al. 2008), and – as will be mentioned later – pilot assays showed little 1F9 epitope specific activity in the Ngerenya October 2002 cross sectional bleed samples.

The prevalence of total AMA-1 antibodies was high in Chonyi at 82.3% (95% CI; 77.6 – 87.1%) with 42.8% of the cohort parasitaemic (by light microscopy) at sample time. When children below the age of 11 years were sampled, most had measurable IgG to AMA-1 (82.3%, 95% CI 77.5 – 87.0%). Antibodies to the 1F9 epitope (measured by the ability of antibodies to inhibit binding of 1F9 to AMA1) were less common in individuals from Chonyi. In this cohort, only 20.4% (95% CI 15.7 – 25.1) for all individuals and 20.7% (95% CI 15.9 – 25.5%) of children under 11 years of age had measureable antibodies to the 1F9-specific epitope. Antibodies to mAbs 2C5 and 5G8 were also measured in Chonyi. Antibodies to these epitopes appeared more prevalent than to the 1F9 epitope in children less than 11 years. The prevalence in children under 11 years was 52.0% (95% CI 45.8 – 58.3%) for 2C5 and 78.6% (95% CI 73.5 – 83.8%) for 5G8.

Antibodies to mAb-specfic epitopes were expressed as the percentage inhibition of the mAb in question. A high level of inhibition therefore implies that there are significant antibodies specific to the epitope in question. The range of antibody reactivity to the 1F9 epitope varied widely in Chonyi; from -14.6 to 65.1%. Negative values of inhibition were suggestive of enhanced binding of the monoclonal antibody to its epitope. Though the Chonyi 2000 cohort also had many

individuals exhibiting low anti-1F9 activity (median 0.4%, -0.41 – 5.4% IQR), the proportion of those with measurable antibodies (20.4%) above the negative controls made this cohort ideal for further study.

Responses to recombinant AMA1, the 1F9 epitope, the 2C5 epitope, and the 5G8 epitope were measured using samples from the Chonyi cohort. The median percent reactivity to the three specific epitopes was varied (1F9 0.4%, 2C5 8.3%, 5G8 7.1%). Reactivity to the mAb 2C5 epitope had the widest range (-18.0% to 93.6%) with 5G8 reactivity exhibiting the narrowest range (-11.3% to 29.7%). Wide ranges of reactivity together with low mean values are indicative of only a few of the individuals in the cohort contributing most of the observed epitope-specific reactivity. Only 13.4% (95% CI, 9.4 – 17.4) of the sera had anti-1f9 activity greater than 10% whereas 44.9% (95% CI 38.8 – 51.1) had anti-2C5 reactivity above 10% and 34.7% (95% CI 28.8-40.5) had anti-5G8 reactivity above 10%.

In the lower transmission setting (Ngerenya), the prevalence of anti AMA-1 IgG antibodies was 58.8% (95% CI 53.2 – 64.5%) and only 6.9% of the individuals were parasitaemic (light microscopy) at sample time. Prevalence of 1F9 epitope reactivity was also lower, with only 15.7% (95% CI 11.5-19.8) of the children positive for inhibitory activity. This cohort (Ngerenya 2002) cohort was not analysed further. Non-malaria exposed individuals were used as negative controls and had no reactivity to the monoclonal antibodies.

7.3.2 Age- and exposure-related acquisition of epitope-specific antibodies

All the samples in the Chonyi cohort were grouped according to age at the time of sampling. Parasite prevalence was below 50% in children between the ages of 1 and 7 years, was highest in the 9 – 10 age groups (90%), and fell to 26% in the older individuals. The prevalence of antibodies to recombinant AMA1 (3D7) was above 50% in all age groups. By the age of 3 years 80% of all children tested positive for antibodies to recombinant AMA1, and the proportion increased with age (test for trend, $p < 0.001$) (Figure 7.2A). By the age of 11 all individuals were anti-AMA1 IgG positive. Maternal antibodies probably explain the high proportion (67%) of children under the age

of 1 with antibodies to the 2C5 epitope (Figure 7.2C). These numbers fell to less than 30% in children ages 1 – 2 years and then rose sharply in the next age group (55%). There continued a gradual increase in numbers with age (test for trend, p= 0.003), up to a maximum value of 70% amongst the 9 – 10 year olds. Antibodies to 1F9 remained less prevalent than those to recombinant AMA1 and the 2C5 epitope (Figure 7.2B). Only 40% of individuals above 10 years tested positive for these antibodies. There was a significant trend ($p < 0.001$) for increasing numbers acquiring anti-1f9 epitope-specific antibodies with age. The proportion of individuals with anti-5G8 epitope-specific antibodies is relatively constant throughout all age groups at around 80% from 3 years onwards (Figure 7.2D).

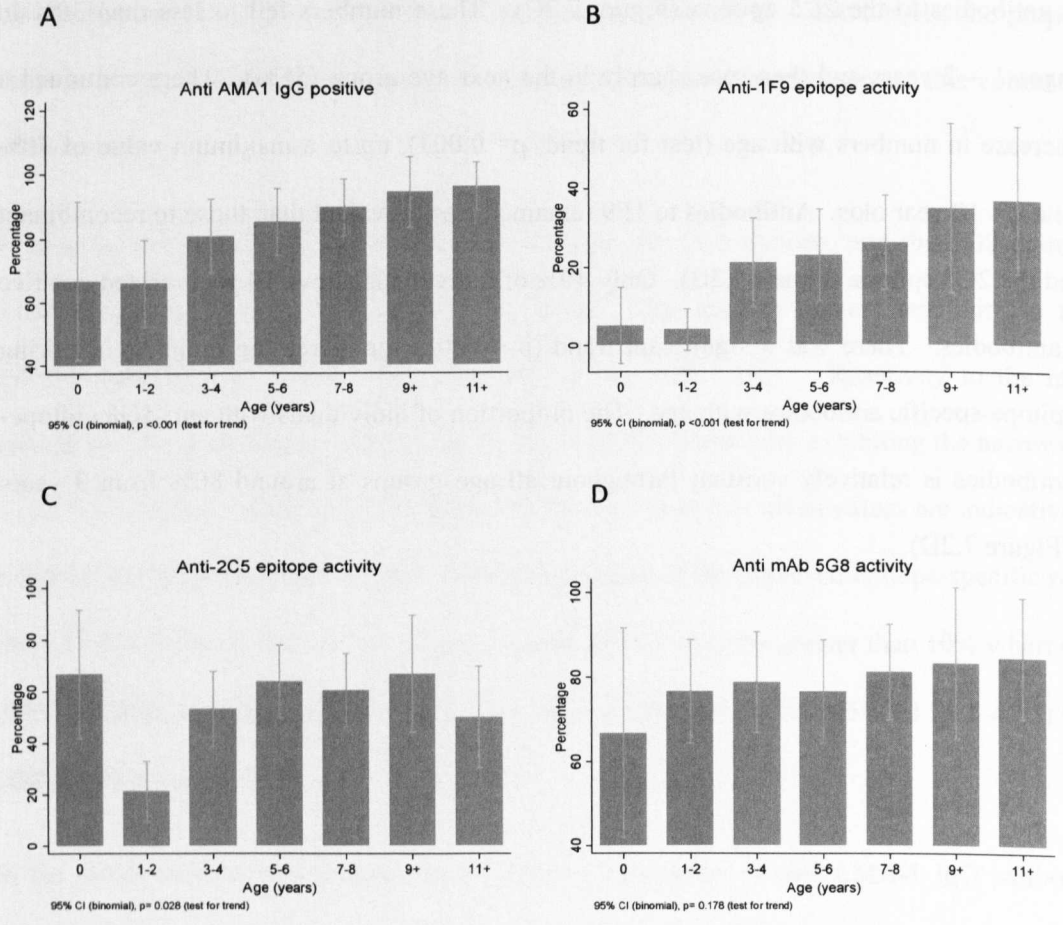

Figure 7.2 Proportion of individuals with dectecatble reactivity to *P.* Falciparum AMA1(3D7) by age group. Antibodies to recombinant AMA1(3D7) tested by standard ELISA. Antibodies to specific AMA1(3D7) epitopes tested by competition ELISA. Error bars indicate 95% CI. Chi^2 test for trend excluding age 0 years. (A) Recombinant AMA1(3D7) $p < 0.001$, (B) 1F9 epitope $p < 0.001$, (C) 2C5 epitope $p = 0.028$, (D) 5G8 epitope $p = 0.178$.

As antibody data was not normally distributed; median antibody levels were analysed within each age group. Acquisition of antibodies to recombinant AMA1 gradually increased with age ($p<0.001$, Kruskal Wallis) though a slight decrease in antibodies in children between ages 1 – 2 years possibly indicating a reduction in maternal antibodies (Figure 7.3A). The association between age and acquisition of anti-2C5 epitope-specific antibodies was not as strong. Though mean levels were significantly higher in children between ages 9 and 10 years compared to those in children between 1 and 2 years, there was no corresponding increase between ages 3 and 9 years of age (Figure 7.3C). The trend of acquisition of antibodies of the 1F9 epitope with age was not as clear. There was a wide range of inhibitory activity in each age group and there was a trend to

higher maximal values with age (not significant). There were negative values for 1F9 epitope reactivity in all the age groups (Figure 7.3B) which was due to the monoclonal antibody binding in greater amounts in the wells. Median antibody reactivity to the 5G8 epitope was similar from ages 1 through to the oldest individuals at around 8% (Figure 7.3D). Children below 1 had slightly lower reactivity but it was not significant.

Presence of *P. falciparum* at the time of sampling was associated with higher antibody levels that are parasite specific, possibly reflecting boosting. This pattern was observed for AMA1, 1F9, and 2C5. Individuals without parasites had significantly lower antibody levels than those with parasites at all ages for AMA1, 2C5, and 1F9. The difference in mean antibody levels between parasitaemic and aparasitaemic individuals to the 5G8 epitope was negligible in most of the age groups.

Reactivity to A4 schizont protein was used as a measure of exposure to blood-stage *P. falciparum*. Schizont reactivity was divided into tertiles to represent low, medium, and high prior exposure. The proportion of individuals with antibodies to recombinant AMA1, the 2C5 epitope, and the 1F9 epitope significantly increased ($p<0.001$, test for trend) with increasing prior exposure to *P. falciparum*. This pattern was not seen with regards to the 5G8 epitope ($p= 0.30$, test for trend).

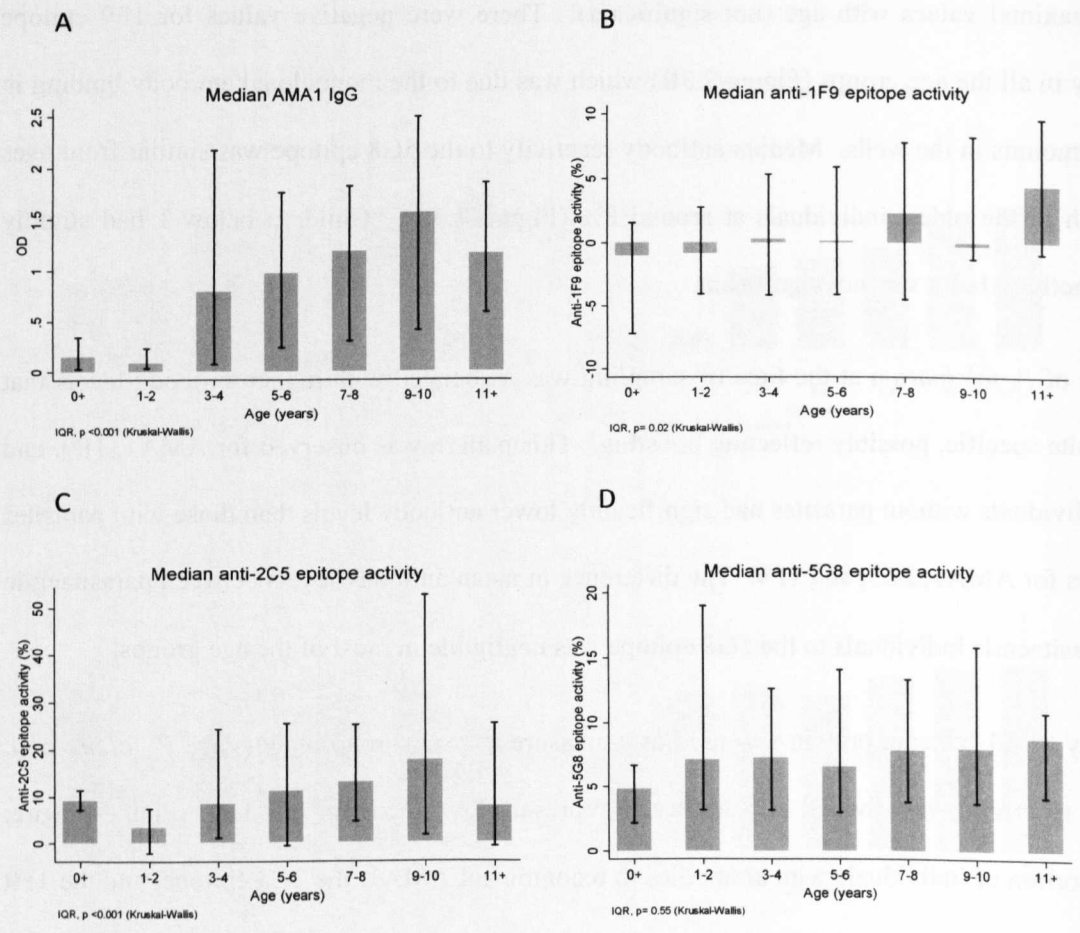

Figure 7.3 Magnitude of the antibody response to recombinant AMA1(3D7) and specific epitopes. Graphs represent median reactivity with error bars indicating the interquartile range. Antibodies to recombinant AMA1(3D7) tested by standard ELISA and represented in absorbance OD units. Antibodies to specific AMA1(3D7) epitopes tested by competition ELISA and represented as percentage activity. Kruskal-Wallis test of equality of medians. (A) Recombinant AMA1(3D7) $p < 0.001$, (B) 1F9 epitope $p = 0.02$, (C) 2C5 epitope $p < 0.001$, (D) 5G8 epitope $p = 0.55$.

Median antibody levels were analysed using these groups. Antibody levels to recombinant AMA1 and the 2C5 epitope increased with increasing schizont protein reactivity ($p < 0.001$, Kruskal-Wallis). Although not as reactive as the response to 2C5, there was also a significant ($p=0.002$, Kruskal-Wallis) increase in antibody levels to the 1F9 epitope with increasing prior exposure to *P. falciparum*. There was a trend to increasing antibody reactivity with increasing prior exposure to the 5G8 epitope for low and medium *P. Falciparum* exposure ($p= 0.04$, Kruskal-Wallis) but it was not as marked as that for the other epitopes.

7.3.3 Relationship between epitope-specific IgG, anti-AMA1 IgG, and IgG responses to schizont protein extract

There was a significant positive correlation ($p<0.001$, Spearmans rank correlation) between reactivity to schizont extract and antibodies to recombinant AMA1 ($r=0.54$), the 1F9 epitope ($r=0.23$), and the 2C5 epitope ($r=0.37$) (Figures 7.4A-C). The relationship between schizont reactivity and 1F9 epitope-specific activity appeared to exhibit threshold characteristics. All 1F9 specific-activity remained below 12% until schizont extract reactivity was above 1 OD unit, then the 1F9 epitope-specific activity increased to 65% (Figure 7.4B). The relationship between schizont extract and 2C5 epitope reactivity exhibited similar threshold characteristics at 1 OD unit (Figure 7.4C). The association between reactivity to schizont extract and to the 5G8 epitope was weak but approached significance ($r=0.13$ $p=0.054$, Spearmans rank correlation) (Figure 7.4D). These results indicate that prior exposure to *P. falciparum* is unlikely to affect the acquisition of antibodies to the 5G8 epitope as expected, since there should be little to exposure to this epitope that is contained in the prodomain.

The association between reactivity to recombinant AMA1 and 1F9 epitope specific reactivity was correlated ($r=0.57$, $p<0.001$ Spearmans rank correlation) (Figure 7.5A). There were several individuals who exhibited 'negative' reactivities to the 1F9 epitope and yet had high levels of antibodies to AMA1, suggesting that some antibodies that bind to AMA1 may enhance binding of 1F9. This association is stronger than that between antibodies to the 1F9 epitope and those to schizont extract. Antibody levels to AMA1 do not always correspond with antibodies to the 1F9 epitope. Individuals with high anti-AMA1 IgG levels (>2.5 OD units) had a range of 1F9 inhibitory activity from 0% to 60%. Conversely, individuals with low antibody reactivity to AMA1 (<0.5 OD units) also exhibited a wide range of 1F9 epitope reactivity (-15% to 40%), though most remained well below 20% (Figure 7.5A). Increased 1F9 binding in the presence of high anti-AMA1 antibodies could be due to conformational changes induced by the anti-AMA1 antibodies leading to enhanced exposure of the 1F9 binding site. Enhanced 1F9 binding in the presence of low anti-AMA1 IgG (<0.5 OD units) may also be due to presence of undetected IgM. Further studies were carried out to investigate this and will be discussed later.

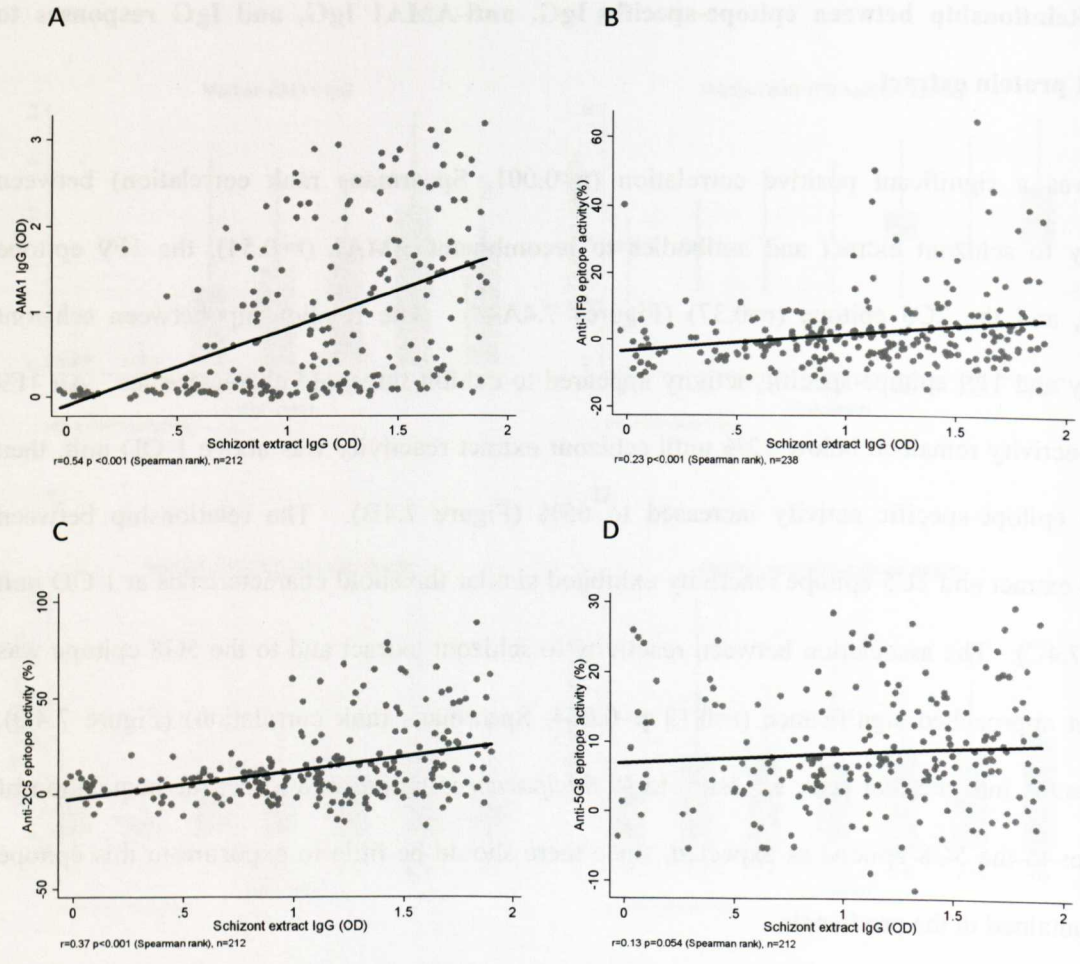

Figure 7.4 Association between antibody reactivity to A4 schizont extract and AMA1(3D7). Spearman rank correlation. (A) schizont extract and recombinant AMA1 ($r = 0.54$; $p < 0.001$), (B) schizont extract and the 1F9 epitope ($r = 0.23$; $p < 0.001$), (C) schizont extract and the 2C5 epitope ($r = 0.37$; $p < 0.001$), (D) schizont extract and the 5G8 epitope ($r = 0.13$; $p = 0.054$).

There was a very strong positive association (r=0.79 p<0.001, Spearmans rank correlation) between antibody reactivity to AMA1 and 2C5 epitope reactivity (Figure 7.5B). High anti-AMA1 IgG levels were associated with high 2C5 epitope specific activity with no obvious outliers. There were far fewer individuals with enhanced 2C5 binding and high anti-AMA1 antibody levels than those observed with 1F9. The association between antibodies to AMA1 and 5G8 epitope reactivity was weak but remained significant (r=0.19 p=0.003, Spearmans rank correlation) (Figure 7.5C). Many individuals with negligible anti-AMA1 antibodies had 5G8 epitope reactivity greater than 10%.

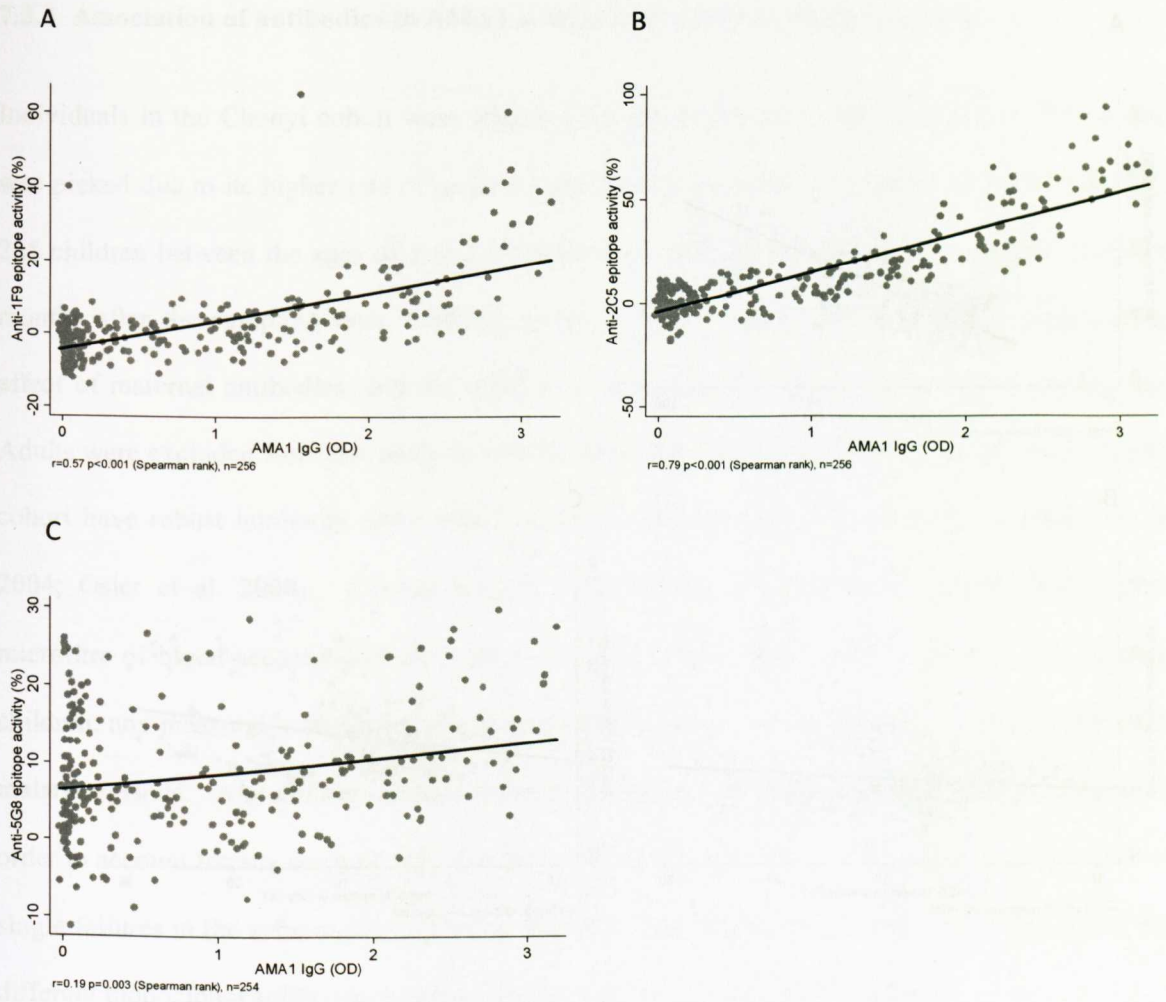

Figure 7.5 Association between antibody reactivity to recombinant AMA1(3D7) and specific epitopes. Spearman rank correlation. (A) Recombinant AMA1 and the 1F9 epitope ($r = 0.57$; $p < 0.001$), (B) recombinant AMA1 and the 2C5 epitope ($r = 0.79$; $p < 0.001$), (C) recombinant AMA1 and the 5G8 epitope ($r = 0.19$; $p = 0.003$).

The correlation between 1F9 epitope and 2C5 epitope antibodies was reasonably strong (r= 0.57 p<0.001, Spearmans rank correlation) and expected, as the 1F9 epitope probably exists within a part of the larger 2C5 epitope, though there were a couple of individuals with high 1F9 epitope reactivity whilst their 2C5 epitope reactivity remained below 25% (Figure 7.6A). There was no association between 1F9 and 5G8 epitope specific reactivity (r=0.02 p=737, Spearmans rank correlation) (Figure 7.6B), and although there was a significant association (p=0.013, Spermans rank correlation) between 5G8 and 2C5 epitope reactivity, it was weak (r= 0.16) (Figure 7.6C).

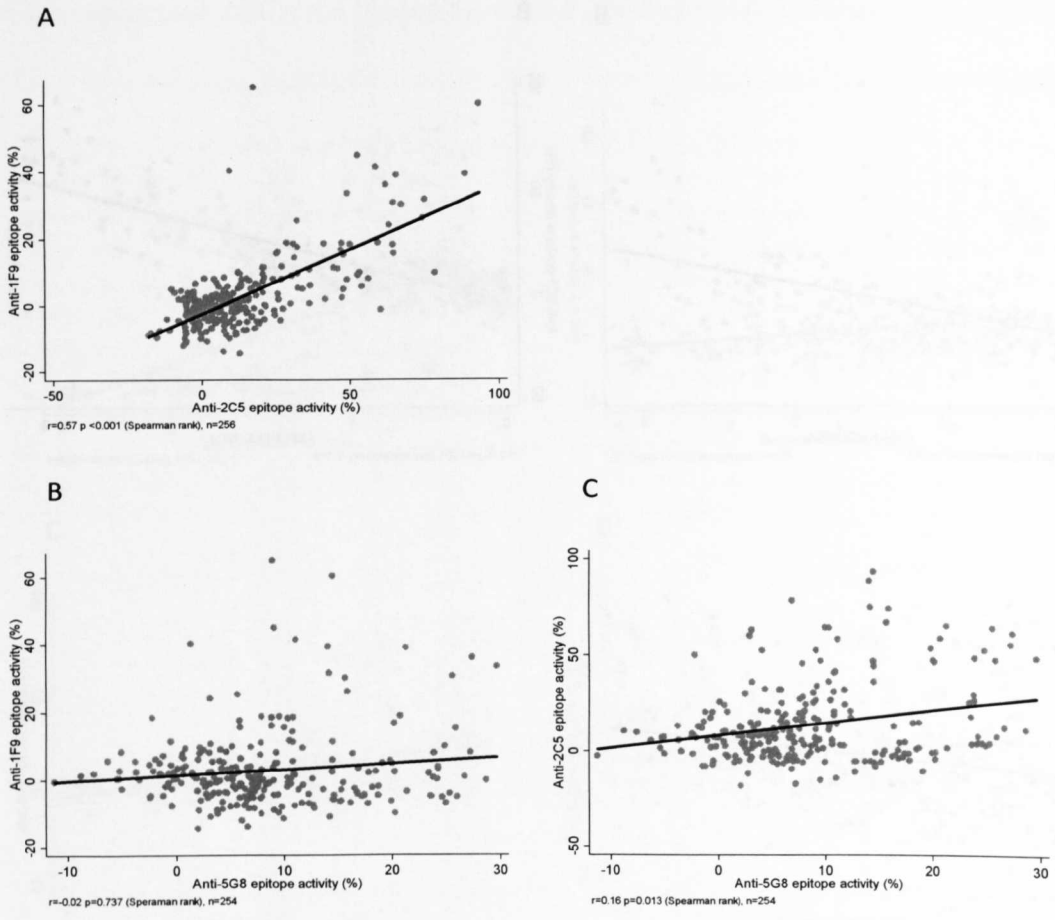

Figure 7.6 Association between antibody reactivity to specific epitopes. Spearman rank correlation. (A) The 2C5 and the 1F9 epitope (r= 0.57; $p < 0.001$), (B) recombinant AMA1 and the 2C5 epitope (r= -0.02; p= 0.737), (C) recombinant AMA1 and the 5G8 epitope (r= 0.16; p= 0.013).

7.3.4 Association of antibodies to AMA1 with protection from clinical malaria

Individuals in the Chonyi cohort were analysed for protection from clinical disease. This cohort was picked due to its higher rate of malaria transmission and higher reactivity to the 1F9 epitope. 228 children between the ages of 1 and 10 years were analysed for episodes of clinical malaria 6 months after the sampling time. Children under 1y were excluded because of the confounding effect of maternal antibodies, and are unlikely to have acquired much immunity before this age. Adults were excluded from this analysis to help reduce the confounding effect of age; adults in this cohort have robust immunity and a broad range of antibodies to different antigens (Polley et al. 2004; Osier et al. 2008). Clinical malaria was defined as parasitaemia >2500 parasites per microlitre of blood accompanied by a fever >37.5°C in individuals >1 year of age. For younger children; any measurable parasitaemia accompanied by a measured fever was considered a clinical malaria episode. Any clinical episodes within 14 days of the sampling date were disregarded in order to account for any ongoing infection at the sampling time. Of the 228 children, there were 34 single failures in the subsequent 6 months. Samples with negative values for competition with the different monoclonal antibodies were not included in this analysis unless stated.

Kaplan-Meier survival curves for the four antibody responses were plotted in order to investigate the protection from clinical malaria further. Individuals were followed up weekly for 6 months after the sampling point and the date of any clinical episodes was recorded. Single failure survival was analysed with censoring of any clinical episode in the first 14 days post sampling. Only three episodes of clinical malaria were disregarded by this censoring. Individuals were categorised according to whether their antibody response was above or below the median as well as by their parasite status at sampling time.

The rate of failure in individuals with a high AMA1 response was similar to that in those with a low response though the overall number of failures in the high group was significantly less ($p=0.013$, Log-rank (Figure 7.7A). When only parasite positive individuals were assessed; a clear difference in the rate of failure was seen. Most individuals who were parasite positive and had a low AMA1 response failed within the first 75 days of follow up compared to those in the high

group (p=0.003, Log-rank) (Figure 7.7B). There was no difference in failure between the high and low AMA1 responders when parasite negative at sampling time.

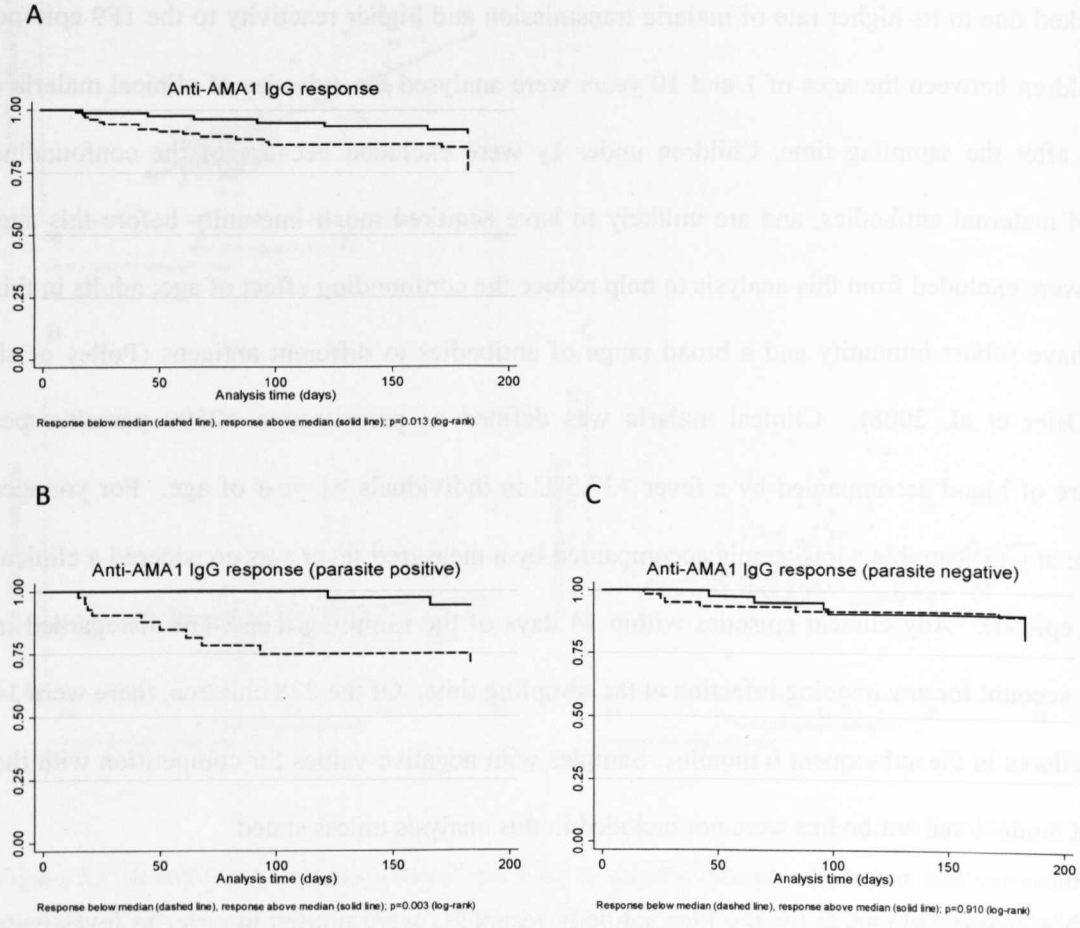

Figure 7.7 Kaplan-Meier estimates for the time to first episode of clinical malaria associated with antibody reactivity to recombinant AMA1(3D7). Graphs show the proportion of individuals remaining free from clinical malaria over time (days). Individuals are categorized according to whether their response to recombinant AMA1 was above (solid line) or below (dashed line) the median reactivity. All samples with negative values for reactivity to specific epitopes were excluded from the analysis. Log-rank test for difference in survival estimates. (A) All individuals (p = 0.013), (B) parasite positive at sampling (p = 0.003), (C) parasite negative at sampling (p = 0.910).

Individuals with a high antibody response to the invasion inhibitory 1F9 epitope had a significantly lower rate of failure compared to those with low responses (p=0.006; Log-rank) (Figure 7.8A). The protective effect of making a high response to the 1F9 epitope appeared to be lost in parasite positive individuals (p=0.12; Log-rank). Though individuals with high levels did not experience any clinical episodes for over 100 days after sampling, their rate of failure in the last 80 days of

follow-up was similar to that experienced by the low responders at the beginning of follow-up (Figure 7.8B). Rates of failure in the parasite negative individuals were not significantly different, though there was only a single failure in the high responders (Figure 7.8C).

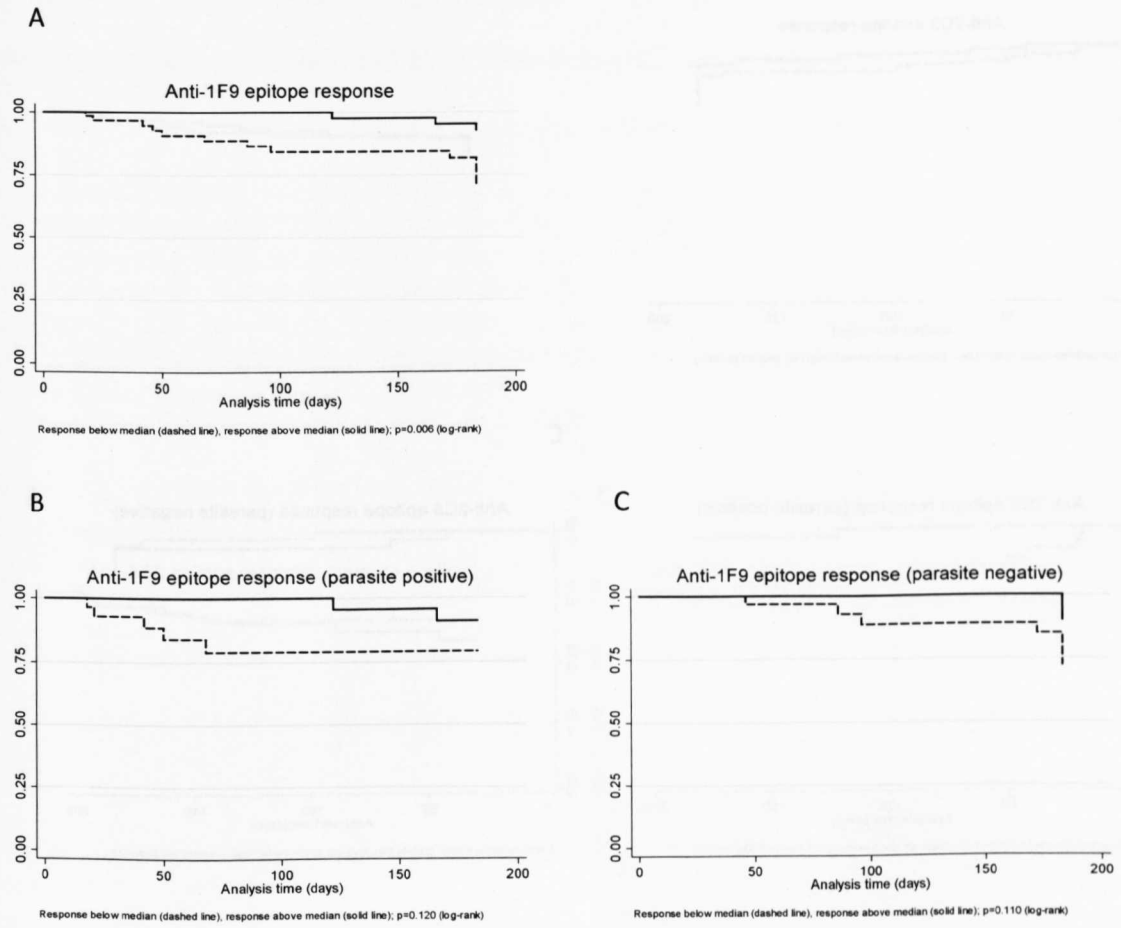

Figure 7.8 Kaplan-Meier estimates for the time to first episode of clinical malaria associated with antibody reactivity to the 1F9 epitope. Graphs show the proportion of individuals remaining free from clinical malaria over time (days). Individuals are categorized according to whether their response to the 1F9 epitope was above (solid line) or below (dashed line) the median reactivity. All samples with negative values for reactivity to specific epitopes were excluded from the analysis. Log-rank test for difference in survival estimates. (A) All individuals (p = 0.006), (B) parasite positive at sampling (p = 0.120), (C) parasite negative at sampling (p = 0.110).

The difference in rates of failure between high and low polymorphic 2C5 epitope responders was marginal (p=0.047; Log-rank) (Figure 7.9A). The greatest difference in rates of failure between these two groups was seen in parasite positive individuals (p=0.003; Log-rank) with the high group rarely failing whereas the low group experienced most of their failures within 100 days of follow-

up (Figure 7.9B). Interestingly, in the parasite negative group; high 2C5 epitope responders had a marginally non-significant higher rate of failure than the low responders (Figure 7.9C).

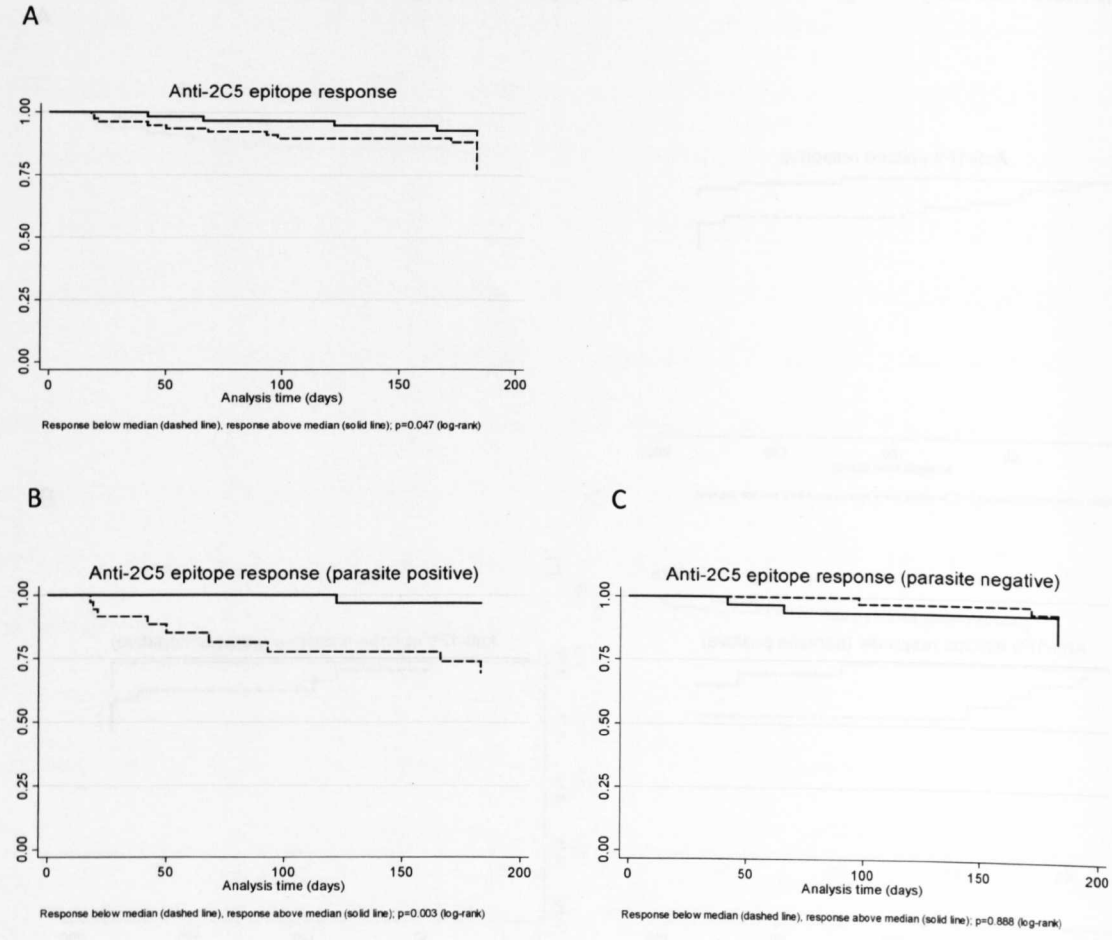

Figure 7.9 Kaplan-Meier estimates for the time to first episode of clinical malaria associated with antibody reactivity to the 2C5 epitope. Graphs show the proportion of individuals remaining free from clinical malaria over time (days). Individuals are categorized according to whether their response to the 2C5 epitope was above (solid line) or below (dashed line) the median reactivity. All samples with negative values for reactivity to specific epitopes were excluded from the analysis. Log-rank test for difference in survival estimates. (A) All individuals ($p = 0.047$), (B) parasite positive at sampling ($p = 0.003$), (C) parasite negative at sampling ($p = 0.888$).

There was no difference in the rates of failure in both high and low 5G8 epitope responders, whether they were parasite positive or negative at sampling time (Figure 7.10A-C).

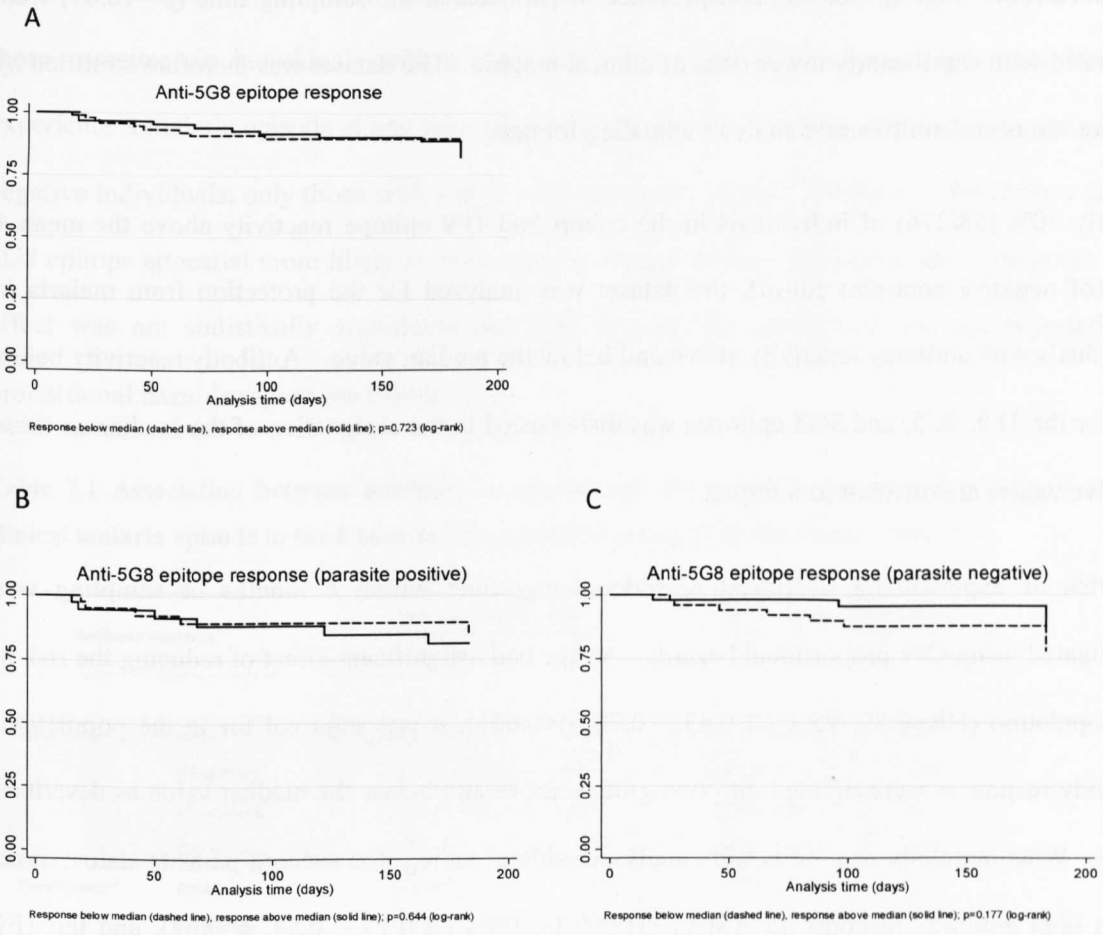

Figure 7.10 Kaplan-Meier estimates for the time to first episode of clinical malaria associated with antibody reactivity to the 5G8 epitope. Graphs show the proportion of individuals remaining free from clinical malaria over time (days). Individuals are categorized according to whether their response to the 5G8 epitope was above (solid line) or below (dashed line) the median reactivity. All samples with negative values for reactivity to specific epitopes were excluded from the analysis. Log-rank test for difference in survival estimates. (A) All individuals ($p = 0.723$), (B) parasite positive at sampling ($p = 0.644$), (C) parasite negative at sampling ($p = 0.177$).

A published study on the same cohort showed an association with protection from clinical malaria in those individuals positive for antibodies to AMA1 and parasitaemic at sample time (Polley et al. 2004). In light this I investigated the effect of parasites at sampling time and age on associations with protection. Age (p <0.001) and presence of parasites at the sampling time (p = 0.04) were associated with significantly lower risks of clinical malaria. The dataset was therefore stratified by parasite status and multivariate analysis adjusting for age.

As only 20% (58/276) of individuals in the cohort had 1F9 epitope reactivity above the mean + 2SD (of negative controls) cut-off, the dataset was analysed for the protection from malaria in individuals with antibody reactivity above and below the median value. Antibody reactivity below zero for the 1F9, 2C5, and 5G8 epitopes was disregarded in the assignation of the median as these negative values are difficult to interpret.

The risk of experiencing a clinical episode at any time within 6 months of sampling was investigated using Cox proportional hazards. As age had a significant effect of reducing the risk in this population (HR=0.55, 95% CI 0.43 – 0.72; p<0.001), it was adjusted for in the population. Antibody responses were divided into two groups above and below the median value as described earlier. When antibody responses were analysed without taking into account parasite status; those with a high antibody response to AMA1 (HR=0.36, 95% CI 0.15 – 0.84; p=0.02), and the 1F9 epitope (HR=0.21, 95% CI 0.06 – 0.73; p=0.01) were significantly less likely to experience a clinical malaria episode at any point during the 6 months after sampling (Table 7.1). Only the low responders to the 1F9 epitope maintained the propensity to experience a clinical episode when the adjusted hazard ratio was calculated, though it was not significant (Adjusted HR=0.34, 95% CI 0.09 – 1.31; p=0.12). High responders to the 2C5 epitope were also less likely to experience a clinical episode at any time. This reduction approached significance (HR=0.38, 95% CI 0.14 – 1.04; p=0.06) but the advantage was lost when the adjusted hazard ratio was calculated. Overall, high responders to the 5G8 epitope were no more protected from experiencing a clinical episode than their counterparts with a lower response.

High responders to AMA1 (HR=0.14, 95% CI 0.03 – 0.64; p=0.01) and to the 2C5 epitope (HR=0.08, 95% CI 0.01 – 0.66; p=0.02) who were parasite positive at sampling time were much

less likely to experience a clinical episode of malaria. They maintained this apparent protection when adjusted for age but the significance diminished (p=0.08) (Table 7.1). Though high responders to the 1F9 epitope who were parasite positive at sampling were less likely to experience a malaria episode with and without age adjustment, it was not a significant effect. Interestingly, those parasitaemic individuals with a high response to the 5G8 epitope were more likely to experience a malaria episode at any time point compared to those with a low response. In parasite negative individuals, only those with a high response to the invasion inhibitory 1F9 epitope and the 5G8 epitope appeared more likely to experience a clinical malaria episode at any time point. This effect was not statistically significant but held in both the unadjusted and age-adjusted Cox proportional hazards regression (Table 7.1).

Table 7.1 Association between antibody responses and the proportional hazard of experiencing a clinical malaria episode in the 6 months subsequent to sampling in the Chonyi cohort

Antibody reactivity[†]		Cox proportional hazards		Adjusted Cox proportional hazards[‡]	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
All samples	AMA1	0.36 (0.15 - 0.84)	**0.02**	0.81 (0.31 - 2.10)	0.66
	IF9 epitope[1]	0.21 (0.06 - 0.73)	**0.01**	0.34 (0.09 - 1.31)	0.12
	2C5 epitope[1]	0.38 (0.14 - 1.04)	**0.06**	0.85 (0.28 - 2.55)	0.77
	5G8 epitope[1]	0.87 (0.40 - 1.88)	0.73	0.96 (0.44 - 2.09)	0.92
Parasitaemic[2]	AMA1	0.14 (0.03 - 0.64)	**0.01**	0.23 (0.05 -1.18)	0.08
	IF9 epitope[1]	0.29 (0.06 - 1.52)	0.14	0.49 (0.09 - 2.74)	0.41
	2C5 epitope[1]	0.08 (0.01 - 0.66)	**0.02**	0.15 (0.02 - 1.27)	**0.08**
	5G8 epitope[1]	1.32 (0.40 - 4.33)	0.65	2.37 (0.68 - 8.27)	0.18
Aparasitaemic[2]	AMA1	0.95 (0.37 - 2.44)	0.91	1.55 (0.58 - 4.16)	0.39
	IF9 epitope[1]	0.31 (0.06 - 1.49)	0.14	0.38 (0.08 - 1.85)	0.23
	2C5 epitope[1]	1.09 (0.33 - 3.56)	0.89	1.36 (0.41 - 4.45)	0.61
	5G8 epitope[1]	0.49 (0.17 - 1.44)	0.19	0.51 (0.17 - 1.50)	0.22

Notes
[†] Antibody reactivity divided into two groups at the median value.
[1] Negative values of activity disregarded in the assignation of the median value.
[‡] Multivariable Cox proportional hazards adjusted for age.
[2] Parasitaemia detetced by light microscopy.
P-values ≤0.1 indicated in bold.

In order to investigate the effect on protection from clinical malaria in those individuals with negative anti-1F9 epitope reactivity new categories were formed for further survival analysis. The three categories were; (1) those with negative anti-1F9 reactivity (i.e. values below zero), (2) those with anti-1F9 reactivity below the median of the positive reactivity (low positive), and (3) those with anti-1F9 reactivity above the median of the positive reactivity (high positive). Kaplan-Meier

survival curves for the three groups were plotted and an unexpected pattern was seen (Figure 7.11). There was a significant difference in the rate of failure between the three groups ($p = 0.025$; Log-rank), with those children who had anti-1F9 reactivity in the low positive group displaying the highest rate of failure. This pattern was clearest in the parasite positive individuals ($p = 0.04$; Log-rank) (Figure 7.11B).

When the risk of experiencing a clinical episode at any time within 6 months of sampling was investigated the low positive group was assigned as the baseline group. Children with high anti-1F9 epitope reactivity were less likely to experience a clinical malaria episode in the follow up time (HR = 0.21, 95% CI 0.06 – 0.74; $p = 0.02$), whereas there was no significant association for those with negative values for 1F9 competition (HR = 0.57; 95% CI 0.28 – 1.18; $p = 0.13$) (Table 7.2). When considered in multivariate analysis (adjusted for age), the apparent protection remained (negative HR = 0.68; high positive HR = 0.41), but was not statistically significant. Univariate analysis showed considerable protection from clinical malaria in children with high positive anti-1F9 reactivity who were parasite positive at sampling time (HR =0.15; 95% CI 0.03 – 0.80; $p = 0.03$). This significance was not held when adjusted for age.

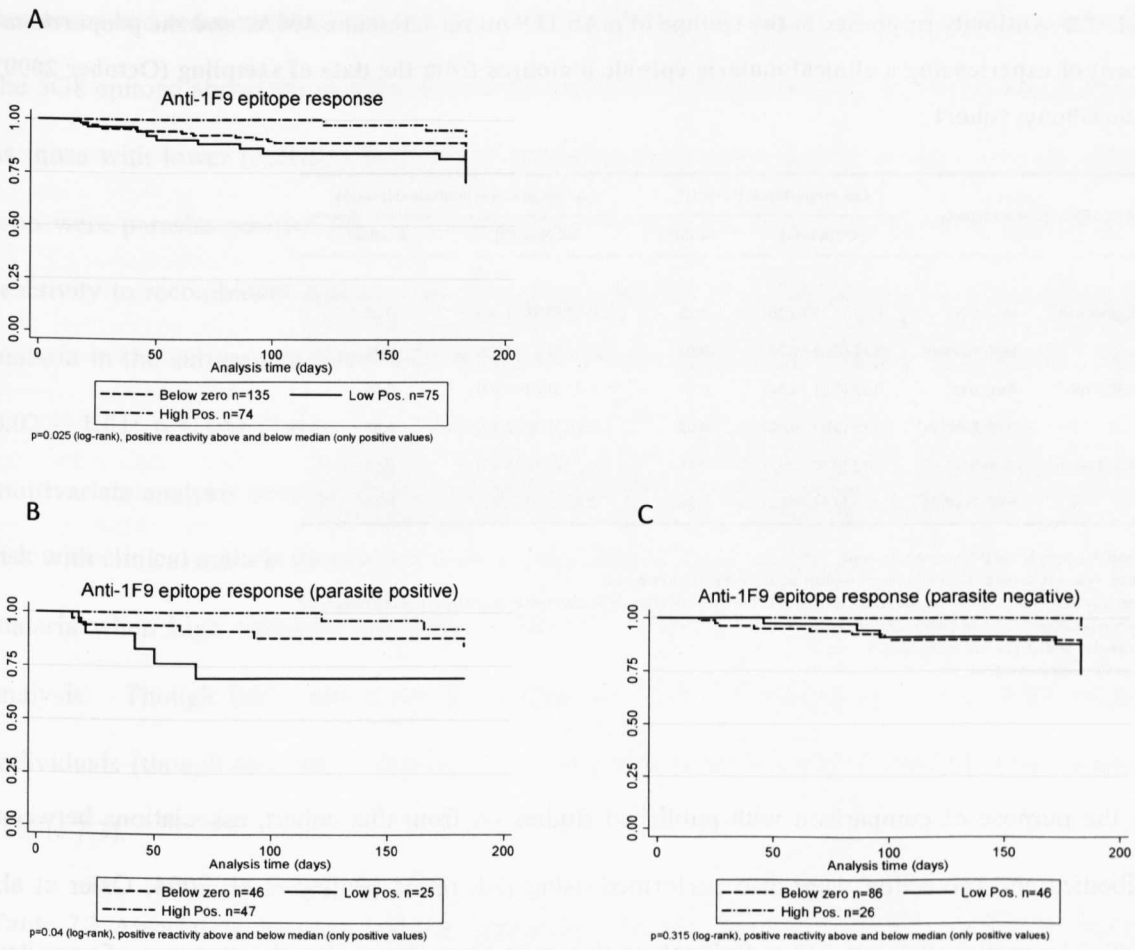

Figure 7.11 Kaplan-Meier estimates for the time to first episode of clinical malaria associated with antibody reactivity to the 1F9 epitope. Graphs show the proportion of individuals remaining free from clinical malaria over time (days). Individuals are categorized according to whether their response to the 1F9 epitope was below zero, below the median reactivity of positive responses (low positive), and above the median reactivity of positive responses (high positive). Log-rank test for difference in survival estimates between the low positive and high positive groups. (A) All individuals (p = 0.025), (B) parasite positive at sampling (p = 0.04), (C) parasite negative at sampling (p = 0.315).

Table 7.2 Antibody responses to the epitope of mAb 1F9 on recombinant AMA1 and the proportional hazard of experiencing a clinical malaria episode 6 months from the date of sampling (October 2000) in the Chonyi cohort

Anti-1F9 Epitope Reactivity		Cox Proportional Hazards[Δ]		Adjusted Cox Proportional Hazards[‡]	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
All individuals	Negative[1]	0.57 (0.28 - 1.18)	0.13	0.68 (0.33 - 1.38)	0.28
	High Positive[2]	0.21 (0.06 - 0.74)	**0.02**	0.41 (0.11 - 1.49)	0.18
Parasitaemic[3]	Negative[1]	0.39 (0.11 - 1.36)	0.14	0.70 (0.18 - 2.62)	0.59
	High Positive[2]	0.15 (0.03 - 0.80)	**0.03**	0.53 (0.09 - 3.15)	0.48
Aparasitaemic[3]	Negative[1]	0.72 (0.30 - 1.73)	0.46	0.77 (0.32 - 1.85)	0.56
	High Positive[2]	0.24 (0.03 - 1.90)	0.18	0.32 (0.04 - 2.62)	0.29

Notes
[1] Antibody reactivity to the 1F9 epitope below zero.
[2] Antibody reactivity to the 1F9 epitope abive te median reactivity of positive responses.
[Δ] Cox proportional hazards in the assigned groups compared to the low positive group (below median reactivity of positive responses).
[‡] Multivariable Cox proportional hazards adjusted for age.
[3] Parasitaemia detetced by light microscopy.
P-values ≤0.1 indicated in bold.

For the purpose of comparison with published studies on from this cohort, associations between antibodies and protection were also performed using risk ratios (Polley et al. 2004; Osier et al. 2008). An earlier study on 537 individuals in this cohort has shown that the presence of parasites and measurable antibody AMA1 (3D7) are associated with protection from clinical malaria (RR=0.33, 95% CI 0.17 – 0.61; p<0.001) (Polley et al. 2004). Univariate logistic regression investigating the risk of developing malaria in similar individuals in this smaller cohort also showed that those individuals with parasites and measurable antibodies were at much lower risk of having a subsequent clinical malaria episode (RR=0.25, 95% CI 0.06 – 1.12; p=0.07). The fewer episodes in this study probably had an effect on the power of the association with protection but the agreement of the trend towards protection in parasite positive individuals was reassuring.

Antibody reactivity to AMA1, the invasion inhibitory 1F9 epitope, and the polymorphic 2C5 epitope above the median decreased the risk of malaria in the subsequent 6 months by univariate analysis (Table 7.3). The risk was significantly reduced with regards to antibodies to AMA1 (RR=0.35, 95%CI 0.16 – 0.78); p=0.01) and antibodies to the 2C5 epitope (RR=0.29, 95%CI 0.11 – 0.78; p=0.01). Though there was also a reduced risk in individuals with reactivity above the median for the 1F9 epitope, it was not statistically significant (RR=0.36, 95%CI 0.1 – 1.27; p=0.11). When looked at in the multivariate analysis (adjusting for age), though all these antibody

responses had reduced risks, none of them were significant. Individuals with antibody reactivity to the 5G8 epitope above the median were at the same risk of experiencing a clinical malaria episode as those with lower reactivity both in the univariate and multivariate analysis. Among children who were parasite positive (by light microscopy) at the time of serum collection, high antibody reactivity to recombinant AMA1, the 1F9 epitope, and the 2C5 epitope conferred protection from malaria in the subsequent 6 months, though this was significant only for 2C5 (RR=0.13, 95%CI 0.02 – 1.01; p=0.05) (Table 7.3). When adjusted for schizont extract reactivity and age in the multivariate analysis only reactivity to AMA1 and the 2C5 epitope were associated with lowered risk with clinical malaria though one were significant. Individuals were at no less risk from clinical malaria when high antibody reactivity to the 1F9 epitope was investigated in the multivariate analysis. Though there was a trend to protection from clinical disease in parasite negative individuals (though to a lesser degree), it was not significant for any of the responses measured (Table 7.3).

Table 7.3 Association between antibody responses and the risk of experiencing a clinical malaria episode in the 6 months subsequent to sampling in the Chonyi cohort

Antibody reactivity[†]		Univariate analysis		Multivariable analysis[‡]	
		RR (95% CI)	*P*-value	RR (95% CI)	*P*-value
All samples	**AMA1**	0.35 (0.16 - 0.78)	**0.01**	0.50 (0.19 - 1.32)	0.16
	1F9 epitope[1]	0.36 (0.10 - 1.27)	0.11	0.56 (0.14 - 2.27)	0.41
	2C5 epitope[1]	0.29 (0.11 - 0.78)	**0.01**	0.40 (0.13 - 1.19)	0.10
	5G8 epitope[1]	1.13 (0.54 - 2.38)	0.75	1.04 (0.46 - 2.36)	0.92
Parasitaemic[2]	**AMA1**	0.25 (0.06 - 1.12)	**0.07**	0.70 (0.11 - 4.45)	0.71
	1F9 epitope[1]	0.52 (0.10 - 2.57)	0.42	1.08 (0.14 - 8.05)	0.94
	2C5 epitope[1]	0.13 (0.02 - 1.01)	**0.05**	0.26 (0.03 - 2.29)	0.23
	5G8 epitope[1]	1.32 (0.43 - 4.09)	0.63	1.04 (0.46 - 2.36)	0.92
Aparasitaemic[2]	**AMA1**	0.47 (0.19 - 1.20)	0.12	0.62 (0.20 - 1.92)	0.41
	1F9 epitope[1]	0.62 (0.13 - 3.00)	0.56	0.81 (0.16 - 4.14)	0.80
	2C5 epitope[1]	0.63 (0.19 - 2.05)	0.44	0.99 (0.26 - 3.72)	0.99
	5G8 epitope[1]	0.84 (0.31 - 2.31)	0.74	0.73 (0.23 - 2.35)	0.60

Notes

[†] Antibody reactivity divided into two groups at the median value.

[1] Negative values of activity disregarded in the assignation of the median value.

[‡] Multivariable analysis adjusted for age and reactivity to schizont extract.

[2] Parasitaemia detetced by light microscopy.

P-values ≤0.1 indicated in bold.

7.3.5 Association of combined antibody responses with protection from clinical malaria

In light of the trend towards protection from clinical malaria in the subsequent analyses and the correlations between anti-AMA1 antibodies and antibodies to the 1F9 epitope ($r=0.57$ $p<0.001$, Spearmans rank), the 2C5 epitope ($r=0.79$ $p<0.001$, Spearmans rank), as well as between the two epitopes ($r=0.57$ $p<0.001$ Spearmans rank); an analysis to define whether those individuals with combined high responses were protected from malaria was carried out. Individuals were assigned as high responders if their antibody response was above the median. Then those with high responses to both AMA1 and the 1F9 epitope, AMA1 and the 2C5 epitope, and both the 1F9 adn 2C5 epitopes were compared to those with combined low responses.

Kaplan-Meier survival curves show that the rate of failure in individuals with a combined high response was significantly lower than that in their low responder counterparts (Figure 7.12). Those with a high AMA1 and high epitope specific response appeared to be better protected (AMA1/1F9 $p=0.006$; AMA1/2C5 $p=0.008$, Log-rank) than those with high 1F9 and 2C5 responses ($p=0.045$, Log-rank). The risk of experiencing a clinical episode at any time within 6 months of sampling was also investigated in these three groupings using Cox proportional hazards. Children with high responses to AMA1 and 1F9 (HR=0.15, 95% CI 0.03 – 0.71; $p=0.02$) as well as those high responses to AMA1 and 2C5 (HR=0.21; 95% CI 0.06 – 0.75; $p=0.02$) were significantly less likely to experience a clinical malaria episode in the follow up time (Table 7.4). Those with high responses to the 1F9 and 2C5 epitopes were also less likely to experience a clinical malaria episode though this was not statistically significant (HR=0.23; 95% CI 0.05 – 1.12; $p=0.07$). When considered in multivariate analysis (adjusted for age), the trend for protection remained but lost its significance for all combinations.

Table 7.4 Combined antibody responses to recombinant and the proportional hazard of experiencing a clinical malaria episode 6 months subsequent to sampling in the Chonyi cohort

Antibody Reactivity Combinations	Cox Proportional hazards		Adjusted Cox proportional hazards[‡]	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
AMA1/1F9	0.15 (0.03 - 0.71)	**0.02**	0.29 (0.05 - 1.61)	0.16
AMA1/2C5	0.21 (0.06 - 0.75)	**0.02**	0.36 (0.08 - 1.50)	0.16
2C5/1F9	0.23 (0.05 - 1.12)	**0.07**	0.34 (0.06 - 2.01)	0.23

Notes

[†] Antibody reactivity above the median (negative values of activity disregarded in the assignation of the median value).

[‡] Multivariable analysis adjusted for age.

P-values ≤0.1 indicated in bold.

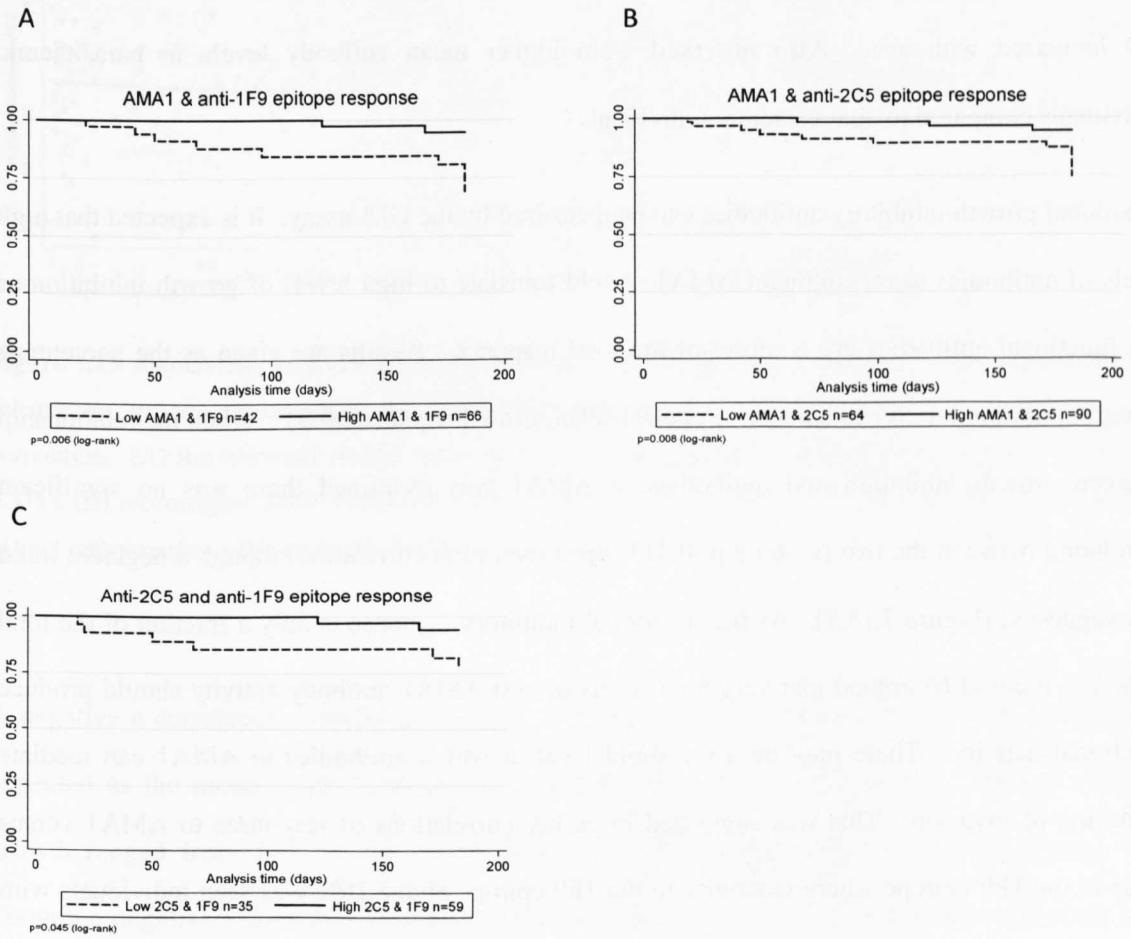

Figure 7.12 Kaplan-Meier estimates for the time to first episode of clinical malaria associated with combined antibody reactivity to recombinant AMA1(3D7) and different epitopes. Graphs show the proportion of individuals remaining free from clinical malaria over time (days). Individuals are categorized according to whether they had high (above the median) or low (below the median) combined responses. All samples with negative values for reactivity to specific epitopes were excluded from the analysis. Log-rank test for difference in survival estimates. (A) Recombinant AMA1 and the 1F9 epitope (p = 0.006), (B) recombinant AMA1 and the 2C5 epitope (p = 0.008), (C) the 1F9 and 2C5 epitopes (p = 0.045).

7.3.6 Association between epitope-specific antibodies and inhibition of *P. falaciparum* growth

A third group of 139 individuals was analysed to investigate the relationship between the 1F9 epitope response and *P. falciparum* growth inhibition *in vitro*; there was insufficient serum available in the Chonyi cohort to perform GIAs. Serum was collected from individuals in Ngerenya during the September 1998 cross-sectional bleed (there was insufficient sera to carry out GIA in the Chonyi cohort) Individuals ranged from 2 – 81 years of age with a mean age of 19.9 years (95% CI 16.3 – 23.5) and a median of 9.4 years. Antibodies to recombinant AMA1, the 1F9 epitope, and inhibition of parasite growth were measured. Median antibody levels to AMA1 and 1F9 increased with age. Also observed were higher mean antibody levels in parasitaemic individuals compared to aparasitaemic individuals.

Functional growth inhibitory antibodies can be measured by the GIA assay. It is expected that high levels of antibodies to recombinant AMA1 should translate to high levels of growth inhibition as any functional antibodies are a subset of the total response. Results are given as the percentage growth inhibition relative to a negative control (PBS, growth equals 100%). When the relationship between growth inhibition and antibodies to AMA1 was examined there was no significant correlation between the two ($r=-0.09$ $p=0.311$, Spearman rank correlation) though a negative trend was suggested (Figure 7.13A). As the functional inhibitory response is only a fraction of the total response, it could be argued that very high levels of anti-AMA1 antibody activity should produce functional activity. There may be a threshold level at which antibodies to AMA1 can mediate inhibition of invasion. This was suggested in earlier correlations of responses to AMA1 versus those to the 1F9 epitope where reactivity to the 1F9 epitope above 10% was seen individuals with high responses to AMA1 (Figure 7.5A).

As the magnitude of the response to AMA1 is strongly associated with increasing prior exposure to *P. falciparum* ($p<0.001$, Kruskal-Wallis), individuals with a low schizont extract antibody response were excluded from further correlations. Exclusion of responses to schizont extract below the median response resulted in strengthening the correlation between inhibition of growth and the antibody response to AMA1. There was a negative correlation ($r=-0.39$ $p=0.001$, Spearmans rank correlation) between the two variables; high antibody levels generally resulting in low parasite

growth *in vitro* (or high levels of inhibition) (Figure 7.13B). However, there were still samples with high levels of AMA1 IgG but little growth inhibitory activity. When this relationship was further investigated by splitting the group into parasite positive and negative individuals, the negative association remained and was slightly strengthened in the parasite positive individuals (r=-0.54 p=0.001, Spearmans rank correlation).

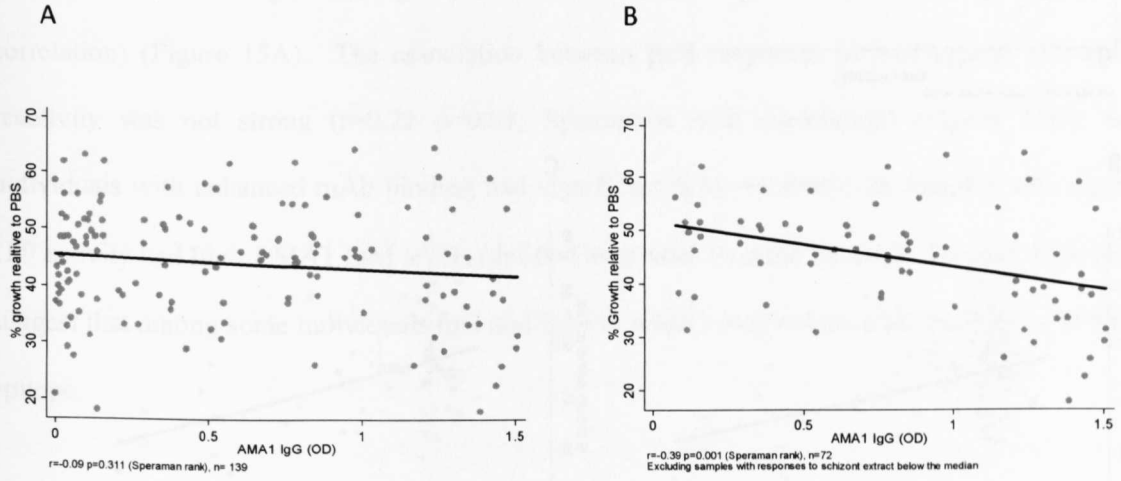

Figure 7.13 Association between antibody reactivity to recombinant AMA1(3D7) and inhibition of *P. falciparum* growth measured by the growth inhibition assay in the Ngerenya cohort. Spearman rank correlation. (A) Recombinant AMA1 antibody reactivity and parasite growth in all samples (r = 0.09; p = 0.311), (B) recombinant AMA1 antibody reactivity and parasite growth in all samples with an A4 schizont extract response above the median (r = -0.39; p = 0.001).

A negative a correlation between parasite growth and antibody reactivity to the 1F9 epitope was expected as the monoclonal antibody (1F9) inhibits parasite growth/invasion. Though parasite growth ranged from 10% – 65%, most of the anti-1F9 epitope activity clustered around 0%. Though a negative association between the two measures was implied, it was not significant (r=-0.11 p=0.239; Spearmans rank correlation) (Figure 7.14B). Exclusion of those individuals with antibody reactivity to AMA1 below the median (as the 1F9 response is a subset of this overall response) resulted in a stronger negative association between parasite growth and 1F9 epitope reactivity (r=-0.25 p=0.042; Spearmans rank correlation) (Figure 7.14C).

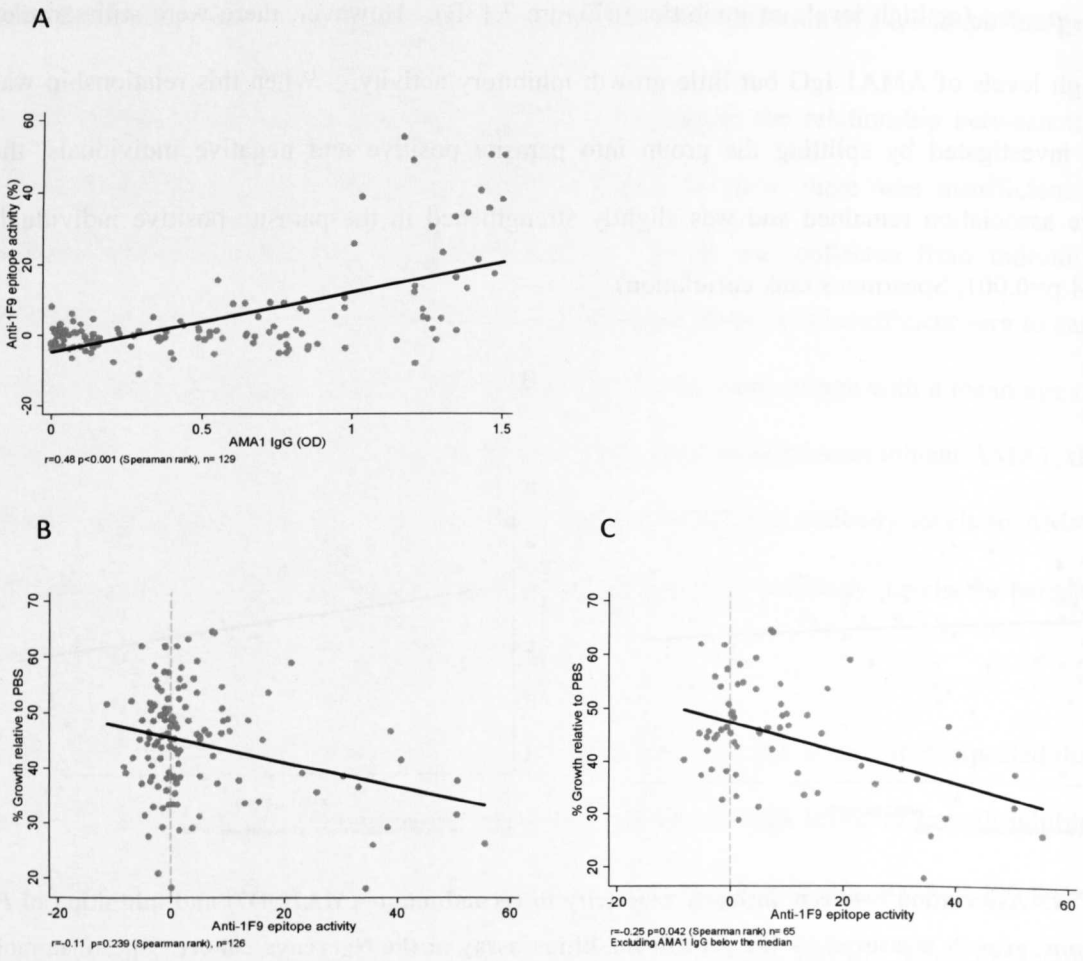

Figure 7.14 Association between antibody reactivity to recombinant AMA1(3D7), 1F9 epitope reactivity, and inhibition of *P. falciparum* growth measured by the growth inhibition assay in the Ngerenya cohort. Spearman rank correlation. (A) Recombinant AMA1 antibody reactivity and 1F9 epitope reactivity (r = 0.48; $p < 0.001$), (B) 1F9 epitope reactivity and parasite growth (r = -0.11; $p = 0.239$), (C) 1F9 epitope reactivity and parasite growth in individuals with AMA1 reactivity above the median value (r = -0.25; $p = 0.042$).

7.3.7 Association between anti-AMA1 IgM and antibodies to specific mAb epitopes

There were a considerable number (n=57) of individuals who appeared to give enhanced binding of the monoclonal 1F9 antibody to its epitope (anti-1F9 epitope activity less than zero). Some of these individuals had significant IgG reactivity to AMA1; 19 samples with negative 1F9 activity had high AMA1 IgG levels (defined as greater than the median) . It is possible these IgG antibodies may bind to other sites on recombinant AMA1 that make the 1F9 epitope more accessible by conformational modification of the recombinant antigen and thereby increase the

binding of 1F9. However, some individuals with enhanced 1F9 binding had low IgG reactivity to AMA1. Therefore, we also investigated whether some individuals have high IgM reactivity to AMA1 that could potentially enhance1F9 binding. IgM levels were measured in the Ngerenya 1998 cohort and analysed for association with 1F9 epitope-specific activity. Few individuals had high IgM responses, which is normal in a malaria endemic population. There was a positive correlation between IgG and IgM reactivity to AMA1 ($r=0.48$ $p<0.001$, Spearmans rank correlation) (Figure 15A). The association between IgM responses to AMA1 and 1F9 epitope reactivity was not strong ($r=0.22$ $p=0.01$, Spearmans rank correlation) (Figure 15B). Some individuals with enhanced mAb binding had significant IgM reactivity; 26 samples with negative 1F9 activity had high AMA1 IgM levels (defined as greater than the median). Togther these results suggest that among some individuals IgG and IgM to AMA1 may enhance the binding of 1F9 to its epitope.

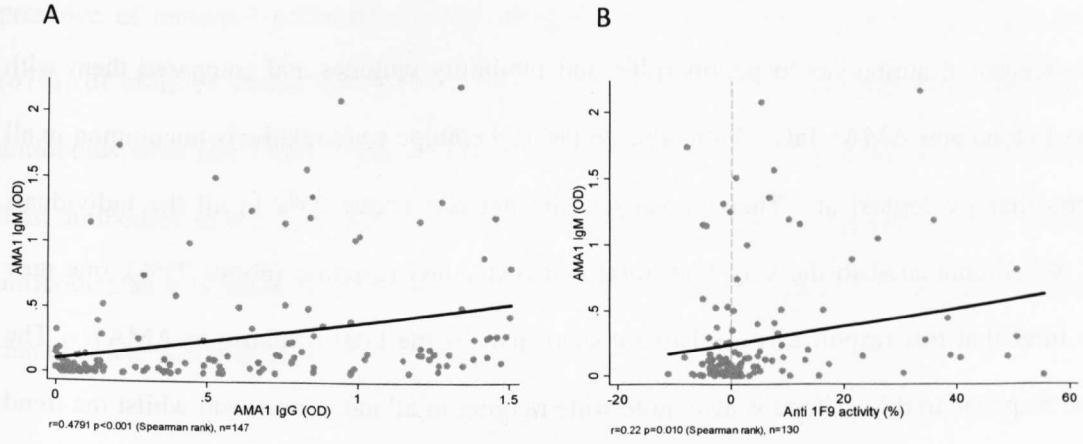

Figure 7.15 Association between IgG and IgM antibody reactivity to recombinant AMA1(3D7), and 1F9 epitope reactivity in the Ngerenya cohort. Spearman rank correlation. (A) Recombinant AMA1 IgG and IgM antibody reactivity ($r = 0.479$; $p = 0.001$), (B) 1F9 epitope reactivity amd recombinant AMA1 IgM antibody reactivity ($r = 0.22$; $p = 0.010$).

7.4 Discussion

AMA1 appears to be highly immunogenic in malaria endemic populations (Thomas et al. 1994; Johnson et al. 2004; Polley et al. 2004; Chelimo et al. 2005; Cortes et al. 2005). As seen, the prevalence of measurable antibodies to AMA1 reaches 80% by the age of 10 in this particular population (Polley et al. 2004). As AMA1 is also under diversifying selection, it is logical that antibodies to this antigen would have some measurable effect on merozoite invasion of the erythrocyte or are relevant to immunity. Both polyclonal and monoclonal antibodies to AMA1 have been shown to be inhibitory to merozoite invasion. Mapping of the epitope of an inhibitory monoclonal antibody has allowed for further investigation on naturally acquired antibodies to AMA1. Monoclonal antibody 1F9 significantly inhibits invasion of 3D7 and D10 *P. falciparum* parasites *in vitro* (Coley et al. 2001; Coley et al. 2006). Whether antibodies to this epitope are abundant and associated with protection in malaria endemic areas was unknown.

Here, we measured antibodies to polymorphic and inhibitory epitopes and compared them with antibodies to total anti-AMA1 IgG. Antibodies to the 1F9 epitope were relatively uncommon in all the cohorts that we looked at. Their prevalence did not rise above 25% in all the individuals studied. When compared to the very high total anti-AMA IgG response (above 75%), one may logically infer that this response is a relatively small part of the total response to AMA1. The individual response to this epitope is also quite wide ranging in all age groups and whilst the trend of increasing levels with age is not as strong as with total anti-AMA1 antibodies, the relationship of increasing levels with increased exposure is significant. The rarity of antibodies to the 1F9 epitope may be explained by the nature of this epitope. Though the antibody footprint is quite large, the majority of the epitope is within a deep hydrophobic cleft (Coley et al. 2007). Binding of other antibodies close by or further away from this site may have conformational effects on this cleft; making it harder to measure antibodies to it. As these are both polymorphic epitopes many children may not have been exposed to them. The affinity of mAbs 1F9 and 2C5 appears to be high (K. Persson, J. Beeson, R. Anders, unpublished data); therefore only those samples with high affinity antibodies to the epitopes may inhibit binding of the respective monoclonal antibodies.

The low prevalence of antibodies to these epitopes could also imply that they are not highly immunogenic.

Antibodies to the mAb 1F9 epitope correlated with increased exposure to *Plasmodium falciparum*, anti-AMA1 antibodies, and antibodies to the 2C5 epitope. Both the increase in antibody prevalence and magnitude of the response to the 1F9 epitope rose with increasing prior exposure to *P. falciparum*. This indicated that though not the 'preferred' antibody response (as overall prevalence is quite low) in this population, recognition of this epitope may confer some protective advantage in malaria endemic settings. As the invasion inhibitory 1F9 epitope is encompassed by the multi-domain polymorphic 2C5 epitope, it was expected that the response to the 2C5 epitope as well as the overall anti-AMA1 response would be positively correlated to that of the 1F9 epitope. Antibodies to the 2C5 epitope (which covers parts of all three domains) were more common than those to the 1F9 epitope, as well as being associated with increasing age and exposure. The presence of maternal antibodies in the children's serum probably explains the high proportion (67%) of children under the age of 1 with antibodies to the polymorphic 2C5 epitope. These antibodies were also more highly correlated with total anti-AMA1 antibodies and schizont extract than antibodies to the 1F9 epitope. The nature of the 2C5 epitope makes it more accessible to antibodies as it is made up of all three domains and does not include the deep hydrophobic cleft that characterises that of 1F9.

High levels of anti-1F9 epitope reactivity are associated with inhibition of erythrocyte invasion by *Plasmodim falciparum* merozoites. In agreement with earlier studies that established the role of specific AMA1 epitopes in erythrocyte invasion inhibition (Coley et al. 2001; Coley et al. 2006), we were able to show that serum with high 1F9 epitope reactivity was more likely to inhibit invasion. Though there was a trend for increasing levels of inhibition with increased 1F9 epitope reactivity the level of inhibition seen was not as high as in other studies. This could be due to the polyclonal nature of the natural immune response; antibodies that block binding to this epitope may be present in the serum of individuals from malaria-endemic areas.

High antibody levels to both polymorphic (2C5) and inhibitory (1F9) epitopes were associated with a reduced risk of clinical disease though this association was not always significant. This indicates that antibodies to these defined epitopes may be involved in contributing to protective immunity. High total IgG levels to recombinant AMA1 were also associated with a reduced risk of clinical disease which is in agreement with published studies on the same cohort (Polley et al. 2004; Osier et al. 2008). It is therefore not surprising that when a combination of antibodies to AMA1 and the 2C5 or 1F9 epitopes was investigated; those children were at a significantly reduced risk of clinical malaria in the 6 months of follow-up. Though the trend of reduced risk remained, it lost significance when adjusted for age with regards to all the antibody responses investigated. As antibodies are associated with age, the loss of significance is not unusual.

In agreement with other studies on the same cohort; the reduced risk of infection was significant in parasite positive rather than slide negative individuals. It has been suggested that a non-humoral component of immunity that is stimulated by circulating parasites may be necessary and will work in concert with the measured antibodies in effectively controlling parasitaemia and thusly reducing the risk of clinical malaria (Polley et al. 2004). Comparison of this study with that of Polley et al, reveals that antibodies to AMA1 (3D7) were two times more protective in our study (RR=0.35 compared to 0.71) in the univariate analysis. There are two differences in our study and methodology that may explain these findings. We compared antibody levels above and below the median whereas Polley et al compared antibody positive and antibody negative individuals. Also, our study was confined to children below the age of 11 whereas Polley et al included adults in their analysis. With an overall reduction in risk of clinical malaria as individuals age the percentage of individuals experiencing malaria in the antibody positive and antibody negative group may not be very different due to intrinsic immune actions honed by both age and cumulative exposure.

This study confirmed the presence of naturally acquired polymorphic and invasion inhibitory antibodies in children from a malaria endemic area, and found they were associated with protection from symptomatic malaria and growth-inhibitory activity by serum antibodies. Presently it is not possible to measure AMA1-specific invasion-inhibitory activity in samples other than by affinity purification of antigen-specific antibodies which requires large sample volumes (typically >20mls are needed). The competition ELISA using MAb 1F9 appears to be a proxy for antibodies to AMA1 inhibitory epitopes and AMA1-specific invasion-inhibitory antibodies. This assay may be valuable for use in AMA1 vaccine trials to indicate the induction of functional and polymorphic antibodies.

8 Concluding Remarks

This study aimed to investigate the dynamics of acquisition and maintenance of antibody responses to *P. falciparum* AMA1 and MSP2 in childhood and examine their role in protective immunity. Investigation of the protective role of antibodies to functional epitopes of AMA1 was an additional aim of this study. Previous studies have shown that antibody responses to merozoite antigens are both short-lived and fluctuant in young children and are affected by malaria transmission levels (Trape 1987; Ramasamy et al. 1994; Jakobsen et al. 1997; Snow et al. 1997; Branch et al. 1998; Marsh and Snow 1999; John et al. 2002; Kinyanjui et al. 2007). Also, longitudinal studies show that antibody levels to different antigens are associated with parasitaemia at the time of sampling or an episode prior to sampling time (Branch et al. 1998; Cavanagh et al. 1998; Giha et al. 1999; Soares et al. 1999; Morais et al. 2006; Biswas et al. 2008). With six cross sectional sampling points over 2.5 years and weekly surveillance of participants, this study was well suited to perform a detailed longitudinal evaluation of acquired immunity and identify factors determining the acquisition and maintenance of antibodies to merozoite antigens and the effects of declining malaria transmission. AMA1 and MSP2 were used as representative merozoite antigens. To facilitate the testing of all samples in the cohort to multiple antigens, a high throughput ELISA using automated robotics was developed and optimised.

Age, prior exposure, and concurrent parasitaemia at sampling were identified as factors affecting the antibody response to AMA1 and MSP2. Malaria transmission levels at the time of sampling not only affected the antibody responses measured, but also the strength of the effect of the above mentioned factors on the overall antibody response. The variability of the effect on antibody levels by age, concurrent parasitaemia, and prior parasitaemia has not been clearly evaluated in the published literature. Here, the decline in malaria transmission allowed for this analysis in a systemic manner and enabled the separation of the effects of age and previous exposure. A model of the effect on antibody responses could then be approximated (Figure 8.1).

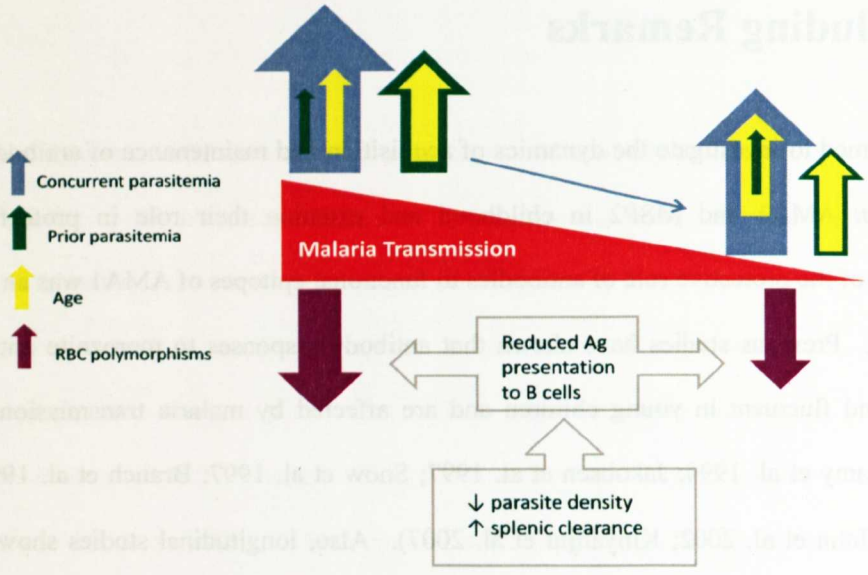

Figure 8.1 Factors affecting antibody responses to AMA1 and MSP2 at different malaria transmission intensities. Antibody levels in parasitaemic and aparasitaemic children are indicated by the relative size of and direction of the arrow. Arrows pointing upwards indicate an increasing effect; arrows pointing downwards indicate a decreasing effect. The contributions of different factors is also indicated by the size of the arrows; blue – concurrent parasitaemia, green – exposure in the six months prior to sampling, yellow – age, and purple – haemoglobinopathies. Reduction in transmission intensity is indicated by the red triangle.

The greatest effect on antibody responses was that of concurrent parasitaemia though previous exposure and age had significant effects. In the case of higher levels of malaria transmission, age had the greatest effect in aparasitaemic children followed by prior exposure; presumably age is reflective of prior exposure. Though both age and prior exposure did have an effect on antibody levels in parasitaemic children, the antibody response appeared to be predominantly related to the presence of active blood-stage parasitaemia. In contrast to the period of higher transmission, age played a much larger role in predicting antibody responses to AMA1 and MSP2 during periods of reduced transmission. In aparasitaemic children, prior exposure played a much larger role in predicting antibody responses together with age. Only when malaria transmission dropped did the antibody response to AMA1 and MSP2 begin to largely reflect cumulative exposure in both parasitaemic and aparasitaemic children. In both periods of higher and lower transmission, both alpha-thalassemia and sickle trait led to lower antibody responses to AMA1 and MSP2 although this reduction was significant

only in a few cases. The sickle trait led to significantly ($p \leq 0.05$) lower antibody responses to all three AMA1 variants tested in 4-6 year olds in May 2003 as well as to the W2mef variant in 1-3 year olds. There was no significant reduction in MSP2 antibody responses. In contrast, alpha-thalassemia homozygotes between the ages of 1-3 year had significantly lower antibody responses to both AMA1 and MSP2 in May 2002 and May 2004 with the same being seen in the oldest children in October 2004. This could be due to reduced immune activation and antigen presentation to B cells as a result of lower parasite densities and/or reduced incidence of clinical malaria episodes associated with those haemoglobinopathies. However it should be noted that although alpha-thalassemia is associated with reduced risk of severe malaria, it has not been consistently associated with reduced parasitaemia or risk of mild malaria.

The probability of a high anti-AMA1 or anti-MSP2 antibody response after recorded exposure in the 6 months prior to sampling increased with increasing exposure up to 3 episodes or did not increase among those with greater exposure. This indicated that a high antibody response was achievable following few exposures and those children who did not exhibit this response may represent non-responders. These children appear to be the more susceptible ones in the population.

Investigation of individual antibody responses to AMA1 and MSP2 over time revealed children with consistently high antibody responses, those with fluctuating antibody responses, and a third group made up of those with consistently low antibody responses. The sampling framework in this study was approximately every 5-6 months which inevitably made it impossible to pick up fine trends in IgG boosting as seen in other studies (Branch et al. 1998; Cavanagh et al. 1998; Kinyanjui et al. 2007; Akpogheneta et al. 2008). This means that the two groups of children described above as those with consistently high or low antibody responses may have included those with fluctuating responses. At a population level, mean antibody levels to all antigens declined in concert with falling malaria transmission rates seen over the same period. Older children always had higher antibody responses without any noticeable increase over time in the antibody responses exhibited by the youngest children. There was a decline in median antibody responses over the study period that implied a strong effect of the declining malaria transmission in the population. Children who had concurrent parasitaemia more

than once at sample time had higher antibody responses throughout the study period compared to those with concurrent parasitaemia only once or never. These results indicated that the antibody responses that were measured in this study were markers of exposure and were maintained by regular exposure to blood-stage parasites. The fact that children with concurrent parasitaemia were 1.5 times more likely to be parasitaemic in the intervals adds to the evidence that antibody levels are a marker of exposure. This study suggests that antibody responses to AMA1 may highly indicative of recent exposure and parallel ongoing changes in malaria transmission.

Antibodies to AMA1 and MSP2 have been associated with protection from malaria in some studies but not in others (Al-Yaman et al. 1994; Al-Yaman et al. 1995; Taylor et al. 1998; Metzger et al. 2003; Polley et al. 2004; John et al. 2005; Polley et al. 2006; Gray et al. 2007; Roussilhon et al. 2007; Nebie et al. 2008; Osier et al. 2008). Even though antibodies contribute a large part of the natural defence against disease, a major issue in understanding immunity to malaria as well as development of vaccines has been the lack of agreement on the protective effects of different antibody responses to malarial antigens. This study showed that antibody responses to AMA1 and MSP2 could be associated with both an increased or decreased risk of malaria in the same cohort of children and that these results were influenced by malaria transmission at sampling and the study framework utilised. In both the cross-sectional and longitudinal analyses, parasitaemia at sampling was associated with an increased risk of disease. The longitudinal association of parasitaemia with increased risk could be indicative of a group of more exposed children. This factor of differences in exposure has been suggested in recent publications (Polley SD et al, Vaccine 2004; Bejon P et al, Infec Imm 2009). This study allowed for identification of the small but significant effect increasing age in childhood had on the reduction of malaria. Although the effect of age is commonly observed by falling rates of parasitaemia and severe illness, its effect has not been identified independently of cumulative exposure. Here, total IgG responses to AMA1(W2mef) were associated with an increased risk of clinical disease whereas those to MSP2(3D7) were associated with possible protection from clinical disease in children.

In both the cross-sectional and longitudinal analyses, parasitaemia at sampling was associated with an increased risk of disease. Exposure to *Plasmodium* can result in three states; symptomatic malaria,

asymptomatic parasitaemia, and lack of detectable parasitaemia. The first is an indicator of an immune system that cannot control the infection and is therefore in the process of acquiring immunity to the infective strain. The second is an indicator of a system with functional immunity to the infective strain. When parasite positivity at baseline response results in protection from future clinical episodes, the implication is that the exposure at baseline was successful in inducing the acquisition of a protective future response. But when the parasitaemia at baseline is accompanied by more disease then the implication is either that the individual cannot mount a protective response, may have a slower rate of acquisition of immunity, or may be exposed to *Plasmodium* strains outside their immune repertoire. Conversely, exposure without resultant parasitaemia would imply a truly protected individual. The above mentioned published results are therefore possibly a snapshot in this acquisition process whereas longitudinal sampling attempts to account for the dynamic process.

IgG1 was the predominant subclass response to AMA1(3D7) in all age groups and throughout the study which is in agreement with published findings (Riley et al. 2000; Polley et al. 2004; Metenou et al. 2007; Nebie et al. 2008; Stanisic et al. 2009). There was little evidence that increasing age led to polarisation of the antibody response to AMA1 as suggested in previous studies (Taylor et al. 1998; Tongren et al. 2006). The longitudinal nature of this study made it particularly valuable at examining whether polarisation of IgG subclass responses occurred with increasing exposure though the reduction in malaria transmission possibly affected this analysis. These findings are consistent with the nature of the antigen being the major determinant of the IgG subclass response (Egan et al. 1995; Taylor et al. 1995; Rzepczyk et al. 1997; Metzger et al. 2003; Tongren et al. 2006; Nebie et al. 2008; Stanisic et al. 2009).

In order to complement the longitudinal studies of antibodies to AMA1, an assay was developed to measure antibodies to functional and polymorphic epitopes of AMA1 using monoclonal antibodies in competition ELISAs. Antibodies to a functional invasion inhibitory epitope of AMA1(3D7), defined by mAb 1F9, were of low prevalence in malaria endemic populations from Kilifi district, including those with high malaria transmission rates. Though antibodies to the mAb 1F9 epitope correlated with increased exposure to *P. falciparum* (as represented by the response to schizont protein extract) they

did not significantly increase with age. High levels of anti-1F9 eptope reactivity were associated with a reduced risk of clinical disease. This study suggests that functional inhibition of erythrocyte invasion by merozoites could be one of the mechanisms involved in protection against malaria and suggest that this assay may be valuable in vaccine trials of AMA1.

A number of factors that may affect antibody responses were not included in this study for various reasons. Exposure to *Plasmodium* is heterogeneous and can be affected by both location (proximity to mosquito breeding sites) and bed net use. Although weekly surveillance gave a strong indication of whether a child had been recently infected, additional information from location and bed net use may have helped properly define those children currently identified as non-exposed. The fact that drug treatment occurred during the study period may have also affected antibody titres. Rapid drug-related clearance of parasites could lead to a lowered antibody response due to reduced exposure of the parasite to the immune system. As anti-malarial medication is widely in use in malaria endemic populations inclusion of the number of times a child received drug treatment may have given a truer picture of the acquisition of antibody responses to AMA1 and MSP2. The above mentioned factors would have assisted in the interpretation of these antibody responses. The underlying effect of each individual's nutritional status would also confound any results obtained in this study. Unfortunately this data was not available.

This study gives compelling evidence of the need for true longitudinal studies (with multiple sampling of antibodies rather than longitudinal surveillance for clinical episodes alone) in the analysis of antibody responses to malaria antigens and their role in protection against malaria. This work could be extended in future studies by measuring responses to additional merozoite antigens, or antigens expressed by other parasite stages, including IgG subclass responses to different antigens. The findings here have significant implications for understanding the targets of protective immunity and the factors determining the acquisition and maintenance of anti-malarial antibodies and immunity. Insights from these studies may be valuable for advancing malaria vaccine development and testing as well as aiding in the development of approaches for measuring exposure to malaria among populations.

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APPENDIX 1

The following appendix includes all results not included in **Chapter 3** but mentioned in the text.

Tables 1–6: IgG responses to AMA1, MSP2, and schizont extract by age group and parasite status at each cross-sectional bleed.

Tables 7–11: IgG responses to AMA1 and MSP2 by reactivity to A4 schizont extract at each cross-sectional bleed.

Tables 12–18: IgG responses to AMA1, MSP2, A4 schizont extract, and tetanus toxoid in the presence or absence of haemoglobinopathies at each cross-sectional bleed.

Tables 19–25: IgG responses to AMA1, MSP2, A4 schizont extract, and tetanus toxoid in the presence and absence of the sickle trait in different age groups.

Tables 26–32: IgG responses to AMA1, MSP2, A4 schizont extract, and tetanus toxoid in the presence and absence of the alpha-thalassemia in different age groups.

Tables 33A – 36F: The effect of different factors on IgG responses to AMA1 and MSP2 at each cross sectional bleed.

Tables 37–39: Correlations between IgG responses to AMA1(HB3), AMA1(3D7), and MSP2(FC27) at each cross sectional bleed.

Table 1 IgG responses to AMA1(W2mef) by age group and parasite status

AMA1(W2mef)		All Samples					Aparasitemic					Parasitemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]
May 2002[1]	N[2]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.04	0.01	0.24	0.24	**<0.001***	0.04	-0.01	0.10	0.44	**<0.001***	0.00	0.65	0.89	1.30	0.710
	IQR	(-0.01 - 0.18)	(-0.02 - 0.17)	(0.03 - 1.18)	(0.1 - 1.9)		(-0.01 - 0.18)	(-0.03 - 0.09)	(0.02 - 0.59)	(0.13 - 1.67)		(0 - 0)	(0.11 - 1.67)	(0.43 - 2.02)	(0.06 - 2.19)	
October 2002[1]	N[2]	37	110	108	36		36	105	99	31		1	5	9	5	
	Median	0.70	0.17	0.66	0.66	**<0.001***	0.73	0.17	0.63	1.60	**<0.001***	-0.04	1.27	1.29	0.86	0.433
	IQR	(0.1 - 1.43)	(0.02 - 0.47)	(0.2 - 1.66)	(0.51 - 2.37)		(0.12 - 1.48)	(0.01 - 0.39)	(0.18 - 1.63)	(0.49 - 2.34)		(-0.04 - -0.04)	(1.13 - 2.2)	(0.27 - 2.19)	(0.66 - 2.47)	
May 2003[1]	N[2]	27	102	105	51		26	93	88	40		1	9	17	11	
	Median	0.01	0.01	0.07	0.07	**<0.001***	0.01	0.00	0.05	0.30	**<0.001***	-0.04	0.04	0.58	2.21	**0.013***
	IQR	(-0.03 - 0.13)	(-0.03 - 0.1)	(0 - 0.73)	(0.08 - 2.06)		(-0.02 - 0.13)	(-0.02 - 0.07)	(0 - 0.46)	(0.07 - 1.51)		(-0.04 - -0.04)	(-0.04 - 0.3)	(0.12 - 1.53)	(0.19 - 2.34)	
October 2003[1]	N[2]	23	103	110	58		23	93	91	45		0	10	18	13	
	Median	0.08	0.02	0.14	0.14	**<0.001***	0.08	0.01	0.07	0.31	**<0.001***	0.00	1.08	1.73	2.19	0.123
	IQR	(0.02 - 0.24)	(-0.01 - 0.1)	(0.02 - 0.78)	(0.07 - 1.92)		(0.02 - 0.24)	(-0.01 - 0.05)	(0.01 - 0.49)	(0.07 - 1.18)		(0 - 0)	(0.21 - 1.71)	(0.44 - 2.13)	(1.61 - 2.26)	
May 2004[1]	N[2]	21	93	86	79		21	88	84	69		0	5	2	10	
	Median	0.00	0.00	0.05	0.05	**<0.001***	0.00	0.00	0.05	0.24	**<0.001***	0.00	1.10	0.22	1.72	0.117
	IQR	(-0.02 - 0.08)	(-0.02 - 0.05)	(0.01 - 0.28)	(0.06 - 1.31)		(-0.02 - 0.08)	(-0.02 - 0.04)	(0.01 - 0.28)	(0.03 - 1.02)		(0 - 0)	(0.58 - 1.55)	(0.1 - 0.34)	(0.98 - 2.03)	
October 2004[1]	N[2]	15	84	87	87		15	80	87	81		0	4	0	6	
	Median	0.02	-0.01	0.03	0.03	**<0.001***	0.02	-0.02	0.03	0.15	**<0.001***	0.00	0.36	0.00	1.12	0.286
	IQR	(-0.01 - 0.21)	(-0.03 - 0.02)	(-0.02 - 0.26)	(0.02 - 0.88)		(-0.01 - 0.21)	(-0.03 - 0.01)	(-0.02 - 0.26)	(0.01 - 0.75)		(0 - 0)	(0.26 - 0.48)	(0 - 0)	(0.2 - 1.5)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

* *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

Table 2 IgG responses to AMA1(HB3) by age group and parasite status.

AMA1(HB3)		All Samples					Aparasitemic					Parasitemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]
May 2002[1]	N[2]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.02	0.00	0.29	0.29	**<0.001***	0.02	-0.02	0.15	0.40	**<0.001***	0.00	0.49	0.91	1.40	0.471
	IQR	(-0.02 - 0.14)	(-0.04 - 0.22)	(0.05 - 1.34)	(0.1 - 1.98)		(-0.02 - 0.14)	(-0.04 - 0.07)	(0.04 - 0.77)	(0.18 - 1.78)		(0 - 0)	(0.12 - 1.87)	(0.39 - 1.99)	(0.09 - 2.17)	
October 2002[1]	N[2]	37	110	108	36		36	105	99	31		1	5	9	5	
	Median	0.21	0.03	0.17	0.17	**<0.001***	0.23	0.02	0.17	0.44	**<0.001***	0.05	0.56	0.59	0.44	0.576
	IQR	(0.05 - 0.5)	(-0.02 - 0.13)	(0.04 - 0.6)	(0.11 - 1.61)		(0.05 - 0.54)	(-0.02 - 0.09)	(0.04 - 0.54)	(0.12 - 1.44)		(0.05 - 0.05)	(0.36 - 1.04)	(0.2 - 1.02)	(0.1 - 2.05)	
May 2003[1]	N[2]	27	102	105	51		26	93	88	40		1	9	17	11	
	Median	0.04	0.03	0.13	0.13	**<0.001***	0.04	0.03	0.11	0.39	**<0.001***	-0.01	0.09	0.66	2.12	**0.022***
	IQR	(-0.01 - 0.21)	(-0.01 - 0.14)	(0.03 - 0.83)	(0.15 - 2.06)		(-0.01 - 0.21)	(-0.01 - 0.13)	(0.03 - 0.51)	(0.15 - 1.64)		(-0.01 - -0.01)	(-0.02 - 0.32)	(0.19 - 1.73)	(0.7 - 2.2)	
October 2003[1]	N[2]	23	103	110	58		23	93	91	45		0	10	18	13	
	Median	0.00	-0.01	0.10	0.10	**<0.001***	0.00	-0.02	0.06	0.22	**<0.001***	0.00	0.76	1.62	2.12	0.127
	IQR	(-0.04 - 0.28)	(-0.05 - 0.13)	(-0.03 - 0.84)	(0.04 - 1.99)		(-0.04 - 0.28)	(-0.05 - 0.04)	(-0.04 - 0.31)	(0.03 - 1.27)		(0 - 0)	(0.36 - 1.98)	(0.35 - 2.06)	(1.33 - 2.18)	
May 2004[1]	N[2]	21	93	86	79		21	88	84	69		0	5	2	10	
	Median	0.02	0.00	0.07	0.07	**<0.001***	0.02	0.00	0.06	0.25	**<0.001***	0.00	0.96	0.35	1.76	0.083
	IQR	(-0.02 - 0.05)	(-0.02 - 0.08)	(0 - 0.33)	(0.05 - 1.29)		(-0.02 - 0.05)	(-0.03 - 0.05)	(0 - 0.32)	(0.03 - 1.09)		(0 - 0)	(0.22 - 1.36)	(0.17 - 0.54)	(0.84 - 2.1)	
October 2004[1]	N[2]	15	84	87	87		15	80	87	81		0	4	0	6	
	Median	-0.02	-0.04	0.02	0.02	**<0.001***	-0.02	-0.05	0.02	0.15	**<0.001***	0.00	0.63	0.00	0.99	0.831
	IQR	(-0.05 - 0.18)	(-0.07 - 0.01)	(-0.04 - 0.29)	(-0.01 - 0.8)		(-0.05 - 0.18)	(-0.07 - -0.01)	(-0.04 - 0.29)	(-0.02 - 0.69)		(0 - 0)	(0.49 - 0.85)	(0 - 0)	(0.24 - 1.86)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

* *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

Table 3 IgG responses to AMA1(3D7) by age group and parasite status.

AMA1(3D7)		All Samples					Aparasitemic					Parasitemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]
May 2002[1]	N[2]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.03	0.02	0.18	0.18	**<0.001***	0.03	0.00	0.11	0.32	**<0.001***	0.00	0.48	0.60	1.11	0.769
	IQR	(0 - 0.08)	(-0.01 - 0.14)	(0.04 - 0.82)	(0.07 - 1.77)		(0 - 0.08)	(-0.02 - 0.06)	(0.03 - 0.61)	(0.07 - 1.48)		(0 - 0)	(0.06 - 1.7)	(0.22 - 1.66)	(0.07 - 2.15)	
October 2002[1]	N[2]	37	110	108	36		36	105	99	31		1	5	9	5	
	Median	0.08	0.00	0.12	0.12	**<0.001***	0.10	0.00	0.11	0.43	**<0.001***	0.02	0.52	0.34	0.36	0.672
	IQR	(0.01 - 0.25)	(-0.02 - 0.07)	(0.02 - 0.38)	(0.05 - 1.54)		(0.01 - 0.27)	(-0.02 - 0.06)	(0.02 - 0.33)	(0.05 - 1.49)		(0.02 - 0.02)	(0.2 - 0.75)	(0.12 - 1.07)	(0.35 - 2.14)	
May 2003[1]	N[2]	27	102	105	51		26	93	88	40		1	9	17	11	
	Median	0.02	0.02	0.10	0.10	**<0.001***	0.03	0.02	0.08	0.37	**<0.001***	-0.02	0.05	0.48	2.16	**0.007***
	IQR	(0 - 0.16)	(-0.01 - 0.08)	(0.02 - 0.5)	(0.1 - 1.99)		(0 - 0.16)	(-0.01 - 0.08)	(0.02 - 0.36)	(0.09 - 1.4)		(-0.02 - -0.02)	(0 - 0.33)	(0.1 - 1.26)	(0.64 - 2.26)	
October 2003[1]	N[2]	23	103	110	58		23	93	91	45		0	10	18	13	
	Median	0.03	0.01	0.09	0.09	**<0.001***	0.03	0.00	0.08	0.29	**<0.001***	0.00	0.40	1.58	2.01	0.090
	IQR	(0 - 0.16)	(-0.02 - 0.11)	(0 - 0.54)	(0.06 - 1.83)		(0 - 0.16)	(-0.02 - 0.07)	(-0.01 - 0.27)	(0.05 - 1.18)		(0 - 0)	(0.14 - 1.54)	(0.24 - 2.02)	(1.72 - 2.24)	
May 2004[1]	N[2]	21	93	86	79		21	88	84	69		0	5	2	10	
	Median	0.00	0.00	0.04	0.04	**<0.001***	0.00	-0.01	0.04	0.21	**<0.001***	0.00	0.54	0.10	1.22	0.112
	IQR	(-0.02 - 0.04)	(-0.02 - 0.05)	(0 - 0.18)	(0.04 - 1.14)		(-0.02 - 0.04)	(-0.02 - 0.05)	(0 - 0.18)	(0.03 - 0.82)		(0 - 0)	(0.19 - 1.19)	(0.07 - 0.14)	(0.67 - 1.77)	
October 2004[1]	N[2]	15	84	87	87		15	80	87	81		0	4	0	6	
	Median	0.05	-0.01	0.03	0.03	**<0.001***	0.05	-0.01	0.03	0.12	**<0.001***	0.00	0.38	0.00	0.59	0.831
	IQR	(-0.02 - 0.11)	(-0.03 - 0.02)	(-0.01 - 0.21)	(0.02 - 0.74)		(-0.02 - 0.11)	(-0.03 - 0.02)	(-0.01 - 0.21)	(0.02 - 0.54)		(0 - 0)	(0.28 - 0.62)	(0 - 0)	(0.18 - 1.32)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

* *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

Table 4 IgG responses to MSP2(3D7) by age group and parasite status.

MSP2(3D7)		All Samples					Aparasitemic					Parasitemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]
May 2002[1]	N[2]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.04	0.13	0.33	0.33	**<0.001***	0.04	0.10	0.28	0.29	**<0.001***	0.00	0.89	1.14	1.76	0.640
	IQR	(0.02 - 0.13)	(0.03 - 0.4)	(0.12 - 1.09)	(0.11 - 1.29)		(0.02 - 0.13)	(0.03 - 0.3)	(0.12 - 0.82)	(0.11 - 0.87)		(0 - 0)	(0.58 - 1.58)	(0.16 - 2.08)	(0.33 - 2.43)	
October 2002[1]	N[2]	37	110	108	36		36	105	99	31		1	5	9	5	
	Median	0.04	0.05	0.14	0.14	**<0.001***	0.04	0.04	0.13	0.12	**<0.001***	0.01	0.62	0.21	1.65	0.193
	IQR	(0.01 - 0.1)	(0.02 - 0.09)	(0.06 - 0.34)	(0.07 - 0.47)		(0.02 - 0.1)	(0.02 - 0.08)	(0.06 - 0.33)	(0.07 - 0.34)		(0.01 - 0.01)	(0.04 - 0.77)	(0.14 - 0.54)	(0.5 - 2.43)	
May 2003[1]	N[2]	27	102	105	51		26	93	88	40		1	9	17	11	
	Median	0.09	0.04	0.13	0.13	**<0.001***	0.10	0.04	0.10	0.14	**<0.001***	0.06	0.12	0.73	1.16	**0.048***
	IQR	(0.01 - 0.23)	(0.01 - 0.1)	(0.05 - 0.43)	(0.07 - 0.72)		(0.01 - 0.23)	(0.01 - 0.09)	(0.04 - 0.31)	(0.06 - 0.31)		(0.06 - 0.06)	(0.04 - 0.26)	(0.2 - 1.39)	(0.2 - 2.56)	
October 2003[1]	N[2]	23	103	110	58		23	93	91	45		10	18	13	0	
	Median	0.08	0.06	0.13	0.13	**<0.001***	0.08	0.06	0.10	0.18	**<0.001***	0.43	0.49	1.77	0.00	0.426
	IQR	(0.04 - 0.19)	(0.02 - 0.25)	(0.05 - 0.41)	(0.08 - 0.56)		(0.04 - 0.19)	(0.01 - 0.16)	(0.04 - 0.28)	(0.07 - 0.44)		(0.29 - 1.03)	(0.32 - 0.75)	(0.32 - 2.17)	(0 - 0)	
May 2004[1]	N[2]	21	93	86	79		21	88	84	69		0	5	2	10	
	Median	0.01	0.04	0.08	0.08	**<0.001***	0.01	0.03	0.08	0.17	**<0.001***	0.00	0.22	0.49	0.43	0.809
	IQR	(0 - 0.02)	(0 - 0.1)	(0.04 - 0.24)	(0.06 - 0.6)		(0 - 0.02)	(0 - 0.09)	(0.04 - 0.23)	(0.04 - 0.47)		(0 - 0)	(0.2 - 1.53)	(0.25 - 0.72)	(0.17 - 2.14)	
October 2004[1]	N[2]	15	84	87	87		15	80	87	81		0	4	0	6	
	Median	0.02	0.04	0.07	0.07	**<0.001***	0.02	0.03	0.07	0.13	**<0.001***	0.00	0.45	0.00	1.63	0.088
	IQR	(0.01 - 0.06)	(0.01 - 0.08)	(0.03 - 0.14)	(0.05 - 0.45)		(0.01 - 0.06)	(0.01 - 0.07)	(0.03 - 0.14)	(0.05 - 0.37)		(0 - 0)	(0.13 - 1.33)	(0 - 0)	(1.17 - 2.31)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

* *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

Table 5 IgG responses to MSP2(FC27) by age group and parasite status.

MSP2(FC27)		All Samples					Aparasitemic					Parasitemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]
May 2002[1]	N[2]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.01	0.04	0.15	0.15	**<0.001***	0.01	0.02	0.10	0.30	**<0.001***	0.00	0.64	0.39	0.40	0.990
	IQR	(-0.01 - 0.1)	(0 - 0.25)	(0.02 - 0.47)	(0.13 - 0.67)		(-0.01 - 0.1)	(-0.01 - 0.18)	(0.01 - 0.36)	(0.11 - 0.61)		(0 - 0)	(0.18 - 1.37)	(0.2 - 1.79)	(0.22 - 0.94)	
October 2002[1]	N[2]	37	110	108	36		36	105	99	31		1	5	9	5	
	Median	0.06	0.03	0.15	0.15	**<0.001***	0.07	0.03	0.13	0.21	**<0.001***	0.00	0.78	0.91	1.81	0.309
	IQR	(0.02 - 0.12)	(0 - 0.13)	(0.03 - 0.48)	(0.09 - 0.73)		(0.02 - 0.12)	(0 - 0.11)	(0.03 - 0.38)	(0.08 - 0.59)		(0 - 0)	(0.71 - 0.97)	(0.35 - 1.84)	(0.53 - 2.38)	
May 2003[1]	N[2]	27	102	105	51		26	93	88	40		1	9	17	11	
	Median	0.05	0.06	0.11	0.11	**<0.001***	0.06	0.05	0.09	0.17	**<0.001***	0.05	0.15	0.63	2.19	**0.006***
	IQR	(0.02 - 0.1)	(0.02 - 0.17)	(0.04 - 0.45)	(0.07 - 0.96)		(0.02 - 0.1)	(0.02 - 0.15)	(0.04 - 0.27)	(0.04 - 0.59)		(0.05 - 0.05)	(0.11 - 0.32)	(0.18 - 1.5)	(0.54 - 2.38)	
October 2003[1]	N[2]	23	103	110	58		23	93	91	45		0	10	18	13	
	Median	0.05	0.05	0.11	0.11	**<0.001***	0.05	0.05	0.09	0.13	**<0.001***	0.00	0.30	0.53	1.50	**0.013***
	IQR	(0.01 - 0.16)	(0.01 - 0.22)	(0.03 - 0.47)	(0.09 - 1.22)		(0.01 - 0.16)	(0.01 - 0.15)	(0.02 - 0.26)	(0.08 - 0.38)		(0 - 0)	(0.21 - 0.88)	(0.22 - 1.48)	(1.2 - 2.37)	
May 2004[1]	N[2]	21	93	86	79		21	88	84	69		0	5	2	10	
	Median	0.01	0.04	0.07	0.07	**<0.001***	0.01	0.04	0.07	0.13	**<0.001***	0.00	0.34	0.13	0.79	0.121
	IQR	(-0.01 - 0.03)	(0.01 - 0.11)	(0.03 - 0.22)	(0.06 - 0.57)		(-0.01 - 0.03)	(0.01 - 0.11)	(0.03 - 0.22)	(0.05 - 0.35)		(0 - 0)	(0.23 - 1.75)	(0.03 - 0.22)	(0.45 - 1.83)	
October 2004[1]	N[2]	15	84	87	87		15	80	87	81		0	4	0	6	
	Median	0.02	0.04	0.06	0.06	**<0.001***	0.02	0.04	0.06	0.12	**<0.001***	0.00	0.40	0.00	0.58	0.522
	IQR	(0.02 - 0.04)	(0.01 - 0.08)	(0.03 - 0.18)	(0.04 - 0.32)		(0.02 - 0.04)	(0.01 - 0.08)	(0.03 - 0.18)	(0.03 - 0.28)		(0 - 0)	(0.28 - 0.66)	(0 - 0)	(0.28 - 1.58)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

* *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

Table 6 IgG responses to schizont extract by age group and parasite status.

Schizont Extract		All Samples					Aparasitemic					Parasitemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]
May 2002[1]	N[2]	30	127	117	23		30	108	93	15		0	19	24	8	
	Median	0.15	0.22	0.60	0.60	**<0.001***	0.15	0.17	0.46	0.64	**<0.001***	0.00	0.94	0.99	1.15	0.520
	IQR	(0.07 - 0.28)	(0.09 - 0.56)	(0.29 - 1.06)	(0.37 - 1.23)		(0.07 - 0.28)	(0.08 - 0.33)	(0.25 - 0.95)	(0.3 - 1.09)		(0 - 0)	(0.73 - 1.21)	(0.64 - 1.53)	(0.87 - 1.23)	
October 2002[1]	N[2]	37	111	108	36		36	105	99	31		1	6	9	5	
	Median	0.07	0.07	0.26	0.26	**<0.001***	0.08	0.06	0.23	0.25	**<0.001***	0.02	0.67	0.86	0.88	0.317
	IQR	(0.02 - 0.21)	(0.02 - 0.15)	(0.09 - 0.57)	(0.13 - 0.89)		(0.03 - 0.21)	(0.02 - 0.13)	(0.08 - 0.53)	(0.11 - 0.7)		(0.02 - 0.02)	(0.38 - 1)	(0.73 - 1.41)	(0.86 - 0.95)	
May 2003[1]	N[2]	27	103	106	52		26	94	89	41		1	9	17	11	
	Median	0.04	0.10	0.19	0.19	**<0.001***	0.03	0.08	0.18	0.47	**<0.001***	0.31	0.60	0.84	1.07	0.153
	IQR	(0.02 - 0.31)	(0.04 - 0.22)	(0.06 - 0.53)	(0.22 - 0.99)		(0.02 - 0.18)	(0.04 - 0.21)	(0.05 - 0.42)	(0.17 - 0.8)		(0.31 - 0.31)	(0.18 - 0.96)	(0.39 - 1.26)	(0.66 - 1.65)	
October 2003[1]	N[2]	21	100	107	57		21	90	88	44		0	10	18	13	
	Median	0.10	0.10	0.26	0.26	**<0.001***	0.10	0.08	0.17	0.41	**<0.001***	0.00	0.99	1.02	1.29	0.293
	IQR	(0.05 - 0.18)	(0.03 - 0.32)	(0.11 - 0.89)	(0.28 - 1.01)		(0.05 - 0.18)	(0.03 - 0.23)	(0.09 - 0.46)	(0.2 - 0.71)		(0 - 0)	(0.76 - 1.37)	(0.68 - 1.26)	(1.05 - 1.39)	
May 2004[1]	N[2]	21	94	87	79		21	89	85	69		0	5	2	10	
	Median	0.22	0.18	0.06	0.06	**<0.001***	0.22	0.18	0.07	0.03	**<0.001***	0.00	0.26	0.03	0.06	0.190
	IQR	(0.09 - 0.39)	(0.08 - 0.38)	(0.02 - 0.28)	(0.01 - 0.1)		(0.09 - 0.39)	(0.08 - 0.38)	(0.02 - 0.28)	(0 - 0.09)		(0 - 0)	(0.14 - 0.33)	(0.02 - 0.05)	(0.02 - 0.56)	
October 2004[1]	N[2]	15	84	87	87		15	80	87	81		0	4	0	6	
	Median	0.03	0.03	0.08	0.08	**<0.001***	0.03	0.03	0.08	0.19	**<0.001***	0.00	0.45	0.00	0.69	0.831
	IQR	(0.01 - 0.08)	(0.01 - 0.06)	(0.04 - 0.18)	(0.1 - 0.43)		(0.01 - 0.08)	(0.01 - 0.06)	(0.04 - 0.18)	(0.1 - 0.35)		(0 - 0)	(0.39 - 0.89)	(0 - 0)	(0.37 - 0.79)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

* *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

Table 7 IgG responses to AMA1(W2mef) by reactivity to A4 schizont extract.

AMA1(W2mef)		Reactivity to Schizont extract			*P*-value[3]
		Low	Medium	High	
May 2002[1]	N[2]	102	100	101	
	Median	-0.01	0.09	0.75	**<0.001**[‡*†]
	IQR	(-0.03 - 0.03)	(0.01 - 0.35)	(0.2 - 1.93)	
October 2002[1]	N[2]	99	98	97	
	Median	0.10	0.39	1.54	**<0.001**[‡*†]
	IQR	(0.01 - 0.29)	(0.16 - 1.01)	(0.73 - 2.37)	
May 2003[1]	N[2]	97	98	95	
	Median	0.01	0.03	0.39	**<0.001**[‡*†]
	IQR	(-0.03 - 0.07)	(-0.01 - 0.18)	(0.06 - 1.7)	
October 2003[1]	N[2]	96	95	95	
	Median	0.01	0.07	1.23	**<0.001**[‡*†]
	IQR	(-0.01 - 0.05)	(0.01 - 0.28)	(0.25 - 2.07)	
May 2004[1]	N[2]	95	93	95	
	Median	0.07	0.04	0.02	**0.020**[‡]
	IQR	(0 - 0.87)	(0 - 0.26)	(-0.01 - 0.23)	
October 2004[1]	N[2]	101	101	100	
	Median	-0.01	0.01	0.49	**<0.001**[‡*†]
	IQR	(-0.03 - 0.01)	(-0.02 - 0.07)	(0.16 - 1.18)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with high schizont reactivity (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with medium schizont reactivity (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with medium and children with high schizont reactivity (*P*-values not shown).

Table 8 IgG responses to AMA1(HB3) by reactivity to A4 schizont extract.

AMA1(HB3)		Reactivity to schizont extract			
		Low	Medium	High	*P*-value[3]
May 2002[1]	N[2]	102	100	101	
	Median	-0.02	0.11	0.83	**<0.001**[‡*†]
	IQR	(-0.04 - 0.03)	(0.01 - 0.37)	(0.25 - 1.87)	
October 2002[1]	N[2]	99	98	97	
	Median	0.02	0.10	0.55	**<0.001**[‡*†]
	IQR	(-0.03 - 0.08)	(0.02 - 0.33)	(0.2 - 1.46)	
May 2003[1]	N[2]	97	98	95	
	Median	0.03	0.08	0.50	**<0.001**[‡*†]
	IQR	(-0.01 - 0.13)	(0.01 - 0.2)	(0.12 - 1.83)	
October 2003[1]	N[2]	96	95	95	
	Median	-0.04	0.06	1.23	**<0.001**[‡*†]
	IQR	(-0.06 - 0.01)	(-0.03 - 0.28)	(0.19 - 2.05)	
May 2004[1]	N[2]	95	93	95	
	Median	0.08	0.05	0.02	0.070[‡]
	IQR	(0 - 0.91)	(-0.01 - 0.25)	(-0.02 - 0.31)	
October 2004[1]	N[2]	101	101	100	
	Median	-0.04	-0.02	0.51	**<0.001**[‡*†]
	IQR	(-0.07 - -0.01)	(-0.05 - 0.08)	(0.15 - 1.24)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with high schizont reactivity (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with medium schizont reactivity (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with medium and children with high schizont reactivity (*P*-values not shown).

Table 9 IgG responses to AMA1(3D7) by reactivity to A4 schizont extract.

AMA1(3D7)		Reactivity to schizont extract			
		Low	Medium	High	*P*-value[3]
May 2002[1]	N[2]	102	100	101	
	Median	0.00	0.07	0.61	**<0.001**[‡*†]
	IQR	(-0.02 - 0.03)	(0.02 - 0.32)	(0.16 - 1.76)	
October 2002[1]	N[2]	99	98	97	
	Median	0.00	0.05	0.38	**<0.001**[‡*†]
	IQR	(-0.02 - 0.04)	(0 - 0.22)	(0.15 - 1.34)	
May 2003[1]	N[2]	97	98	95	
	Median	0.02	0.04	0.45	**<0.001**[‡†]
	IQR	(0 - 0.09)	(0 - 0.17)	(0.07 - 1.68)	
October 2003[1]	N[2]	96	95	95	
	Median	-0.01	0.08	0.96	**<0.001**[‡*†]
	IQR	(-0.02 - 0.03)	(0 - 0.23)	(0.18 - 1.97)	
May 2004[1]	N[2]	95	93	95	
	Median	0.06	0.05	0.02	**0.016**[‡]
	IQR	(0 - 0.74)	(-0.01 - 0.22)	(-0.02 - 0.18)	
October 2004[1]	N[2]	101	101	100	
	Median	-0.01	0.01	0.36	**<0.001**[‡*†]
	IQR	(-0.03 - 0.02)	(-0.01 - 0.07)	(0.11 - 0.95)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with high schizont reactivity (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with medium schizont reactivity (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with medium and children with high schizont reactivity (*P*-values not shown).

Table 10 IgG responses to MSP2(3D7) by reactivity to A4 schizont extract.

MSP2(3D7)		Reactivity to schizont extract			
		Low	Medium	High	*P*-value[3]
May 2002[1]	N[2]	102	100	101	
	Median	0.06	0.16	0.86	**<0.001**[‡*†]
	IQR	(0.02 - 0.18)	(0.05 - 0.42)	(0.35 - 1.81)	
October 2002[1]	N[2]	99	98	97	
	Median	0.03	0.07	0.26	**<0.001**[‡*†]
	IQR	(0.01 - 0.07)	(0.03 - 0.14)	(0.1 - 0.68)	
May 2003[1]	N[2]	97	98	95	
	Median	0.03	0.07	0.26	**<0.001**[‡*†]
	IQR	(0.01 - 0.09)	(0.03 - 0.15)	(0.1 - 0.89)	
October 2003[1]	N[2]	96	95	95	
	Median	0.04	0.13	0.41	**<0.001**[‡*†]
	IQR	(0.01 - 0.08)	(0.05 - 0.25)	(0.17 - 0.91)	
May 2004[1]	N[2]	95	93	95	
	Median	0.12	0.07	0.05	**0.012**[‡]
	IQR	(0.03 - 0.36)	(0.02 - 0.18)	(0.01 - 0.2)	
October 2004[1]	N[2]	101	101	100	
	Median	0.03	0.06	0.21	**<0.001**[‡*†]
	IQR	(0.01 - 0.06)	(0.02 - 0.12)	(0.09 - 0.51)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with high schizont reactivity (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with medium schizont reactivity (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with medium and children with high schizont reactivity (*P*-values not shown).

Table 11 IgG responses to MSP2(FC27) by reactivity to A4 schizont extract.

MSP2(FC27)		Reactivity to schizont extract			
		Low	Medium	High	*P*-value[3]
May 2002[1]	N[2]	102	100	101	
	Median	0.01	0.06	0.56	**<0.001**[‡*†]
	IQR	(-0.02 - 0.06)	(0.01 - 0.22)	(0.2 - 1.6)	
October 2002[1]	N[2]	99	98	97	
	Median	0.03	0.06	0.40	**<0.001**[‡*†]
	IQR	(0 - 0.07)	(0.02 - 0.15)	(0.17 - 0.85)	
May 2003[1]	N[2]	97	98	95	
	Median	0.04	0.07	0.32	**<0.001**[‡*†]
	IQR	(0.01 - 0.09)	(0.03 - 0.17)	(0.14 - 0.96)	
October 2003[1]	N[2]	96	95	95	
	Median	0.03	0.08	0.46	**<0.001**[‡*†]
	IQR	(0.01 - 0.08)	(0.02 - 0.16)	(0.18 - 1.4)	
May 2004[1]	N[2]	95	93	95	
	Median	0.07	0.06	0.05	0.208
	IQR	(0.03 - 0.34)	(0.02 - 0.15)	(0.01 - 0.18)	
October 2004[1]	N[2]	101	101	100	
	Median	0.03	0.04	0.19	**<0.001**[‡*†]
	IQR	(0.01 - 0.05)	(0.02 - 0.11)	(0.09 - 0.41)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with high schizont reactivity (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with medium schizont reactivity (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with medium and children with high schizont reactivity (*P*-values not shown).

Table 12 IgG responses to AMA1(W2mef) in the presence or absence of hemoglobinopathies.

AMA1(W2mef)		Sickle trait			Alpha-Thalassemia			
		HbAA	HBAS	*P*-value[3]	Norm	Het	Homo	*P*-value[4]
May 2002[1]	N[2]	258	41		93	152	52	
	Median	0.09	0.05	0.215	0.09	0.09	0.07	0.521
	IQR	(-0.01 - 0.72)	(0 - 0.23)		(-0.01 - 0.72)	(0 - 0.72)	(0 - 0.25)	
October 2002[1]	N[2]	258	34		92	138	54	
	Median	0.44	0.36	0.141	0.40	0.50	0.21	**0.042**[†]
	IQR	(0.11 - 1.47)	(0.05 - 0.98)		(0.1 - 1.36)	(0.16 - 1.53)	(0.04 - 0.66)	
May 2003[1]	N[2]	246	37		90	137	52	
	Median	0.06	0.01	**0.012**	0.05	0.07	0.01	**0.04**[†]
	IQR	(0 - 0.69)	(-0.03 - 0.21)		(-0.01 - 0.44)	(0 - 0.73)	(-0.02 - 0.1)	
October 2003[1]	N[2]	246	38		97	136	52	
	Median	0.07	0.09	0.908	0.09	0.09	0.03	0.067[*]
	IQR	(0 - 0.69)	(0.02 - 0.24)		(0.02 - 0.85)	(0.01 - 0.58)	(-0.01 - 0.21)	
May 2004[1]	N[2]	231	35		88	124	49	
	Median	0.05	0.04	0.584	0.06	0.05	0.02	**0.029**[*†]
	IQR	(0 - 0.46)	(0 - 0.17)		(0.01 - 0.78)	(0 - 0.41)	(-0.01 - 0.09)	
October 2004[1]	N[2]	232	38		92	126	50	
	Median	0.03	0.03	0.905	0.04	0.03	0.01	0.401
	IQR	(-0.02 - 0.3)	(-0.02 - 0.21)		(-0.01 - 0.35)	(-0.02 - 0.34)	(-0.02 - 0.17)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Wilcoxon rank test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children heterozygous for α-thalassemia (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children homozygous for α-thalassemia (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children heterozygous and homozygous for α-thalassemia (*P*-values not shown).

Table 13 IgG responses to AMA1(HB3) in the presence or absence of hemoglobinopathies.

AMA1(HB3)		Sickle trait			Alpha-Thalassemia			
		HbAA	HBAS	*P*-value[3]	Norm	Het	Homo	*P*-value[4]
May 2002[1]	N[2]	258	41		93	152	52	
	Median	0.10	0.05	0.258	0.09	0.12	0.05	0.169
	IQR	(-0.01 - 0.75)	(-0.02 - 0.3)		(-0.03 - 0.85)	(0 - 0.65)	(-0.02 - 0.27)	
October 2002[1]	N[2]	258	34		92	138	54	
	Median	0.10	0.08	0.454	0.11	0.12	0.04	**0.009**[*†]
	IQR	(0.02 - 0.5)	(0.01 - 0.38)		(0.02 - 0.47)	(0.03 - 0.5)	(-0.02 - 0.19)	
May 2003[1]	N[2]	246	37		90	137	52	
	Median	0.12	0.03	**0.013**	0.11	0.14	0.04	**0.026**[†]
	IQR	(0.02 - 0.79)	(0 - 0.3)		(0 - 0.58)	(0.03 - 0.83)	(0 - 0.14)	
October 2003[1]	N[2]	246	38		97	136	52	
	Median	0.04	0.08	0.684	0.13	0.05	-0.01	0.068[*]
	IQR	(-0.04 - 0.75)	(-0.03 - 0.2)		(-0.02 - 0.91)	(-0.04 - 0.66)	(-0.04 - 0.17)	
May 2004[1]	N[2]	231	35		88	124	49	
	Median	0.06	0.03	0.511	0.09	0.05	0.02	0.058[*]
	IQR	(-0.01 - 0.53)	(0 - 0.2)		(0 - 0.79)	(-0.01 - 0.57)	(-0.02 - 0.11)	
October 2004[1]	N[2]	232	38		92	126	50	
	Median	0.01	0.03	0.68	0.03	0.02	-0.02	0.344
	IQR	(-0.05 - 0.31)	(-0.05 - 0.28)		(-0.04 - 0.39)	(-0.05 - 0.29)	(-0.04 - 0.24)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Wilcoxon rank test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children heterozygous for α-thalassemia (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children homozygous for α-thalassemia (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children heterozygous and homozygous for α-thalassemia (*P*-values not shown).

Table 14 IgG responses to AMA1(3D7) in the presence or absence of hemoglobinopathies.

AMA1(3D7)		Sickle trait			Alpha-Thalassemia			
		HbAA	HBAS	*P*-value[3]	Norm	HbAA	HBAS	*P*-value[4]
May 2002[1]	N[2]	258	41		93	152	52	
	Median	0.07	0.04	0.162	0.06	0.08	0.04	0.357
	IQR	(0 - 0.6)	(0 - 0.15)		(-0.01 - 0.59)	(0.01 - 0.51)	(0.01 - 0.21)	
October 2002[1]	N[2]	258	34		92	138	54	
	Median	0.06	0.05	0.368	0.05	0.09	0.02	0.051[*†]
	IQR	(0 - 0.33)	(0 - 0.19)		(0 - 0.31)	(0 - 0.38)	(-0.01 - 0.14)	
May 2003[1]	N[2]	246	37		90	137	52	
	Median	0.08	0.02	**0.02**	0.06	0.08	0.03	**0.02**[†]
	IQR	(0.01 - 0.6)	(-0.01 - 0.24)		(0 - 0.5)	(0.01 - 0.65)	(-0.01 - 0.13)	
October 2003[1]	N[2]	246	38		97	136	52	
	Median	0.08	0.07	0.843	0.10	0.08	0.02	0.07[*]
	IQR	(-0.01 - 0.46)	(0.01 - 0.19)		(0 - 0.74)	(0 - 0.45)	(-0.01 - 0.13)	
May 2004[1]	N[2]	231	35		88	124	49	
	Median	0.04	0.04	0.47	0.07	0.04	0.00	**0.032**[*†]
	IQR	(-0.01 - 0.36)	(-0.01 - 0.14)		(-0.01 - 0.6)	(-0.01 - 0.36)	(-0.01 - 0.08)	
October 2004[1]	N[2]	232	38		92	126	50	
	Median	0.02	0.04	0.728	0.03	0.04	0.02	0.721
	IQR	(-0.01 - 0.22)	(-0.02 - 0.18)		(-0.01 - 0.27)	(-0.02 - 0.24)	(-0.01 - 0.18)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Wilcoxon rank test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children heterozygous for α-thalassemia (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children homozygous for α-thalassemia (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children heterozygous and homozygous for α-thalassemia (*P*-values not shown).

Table 15 IgG responses to MSP2(3D7) in the presence or absence of hemoglobinopathies.

MSP2(3D7)		Sickle trait			Alpha-Thalassemia			
		HbAA	HBAS	*P*-value[3]	Norm	Het	Homo	*P*-value[4]
May 2002[1]	N[2]	258	41		93	152	52	
	Median	0.24	0.13	0.141	0.26	0.23	0.12	0.059
	IQR	(0.05 - 0.69)	(0.02 - 0.58)		(0.07 - 0.68)	(0.05 - 0.78)	(0.03 - 0.34)	
October 2002[1]	N[2]	258	34		92	138	54	
	Median	0.07	0.09	0.884	0.08	0.08	0.07	0.633
	IQR	(0.03 - 0.23)	(0.03 - 0.17)		(0.03 - 0.25)	(0.03 - 0.26)	(0.03 - 0.16)	
May 2003[1]	N[2]	246	37		90	137	52	
	Median	0.09	0.07	0.4	0.10	0.09	0.07	0.762
	IQR	(0.03 - 0.29)	(0.02 - 0.2)		(0.02 - 0.33)	(0.03 - 0.26)	(0.03 - 0.2)	
October 2003[1]	N[2]	246	38		97	136	52	
	Median	0.13	0.09	0.626	0.15	0.10	0.09	0.487
	IQR	(0.03 - 0.36)	(0.05 - 0.17)		(0.04 - 0.39)	(0.04 - 0.35)	(0.03 - 0.31)	
May 2004[1]	N[2]	231	35		88	124	49	
	Median	0.08	0.07	0.517	0.08	0.07	0.08	0.779
	IQR	(0.02 - 0.26)	(0.02 - 0.22)		(0.03 - 0.29)	(0.02 - 0.27)	(0.03 - 0.17)	
October 2004[1]	N[2]	232	38		92	126	50	
	Median	0.07	0.07	0.895	0.07	0.08	0.06	0.738
	IQR	(0.02 - 0.22)	(0.04 - 0.17)		(0.03 - 0.26)	(0.02 - 0.23)	(0.03 - 0.21)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Wilcoxon rank test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children heterozygous for α-thalassemia (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children homozygous for α-thalassemia (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children heterozygous and homozygous for α-thalassemia (*P*-values not shown).

Table 16 IgG responses to MSP2(FC27) in the presence or absence of hemoglobinopathies.

MSP2(FC27)		Sickle trait			Alpha-Thalassemia			
		HbAA	HBAS	*P*-value[3]	Norm	Het	Homo	*P*-value[4]
May 2002[1]	N[2]	258	41		93	152	52	
	Median	0.11	0.01	0.052	0.14	0.09	0.06	0.397
	IQR	(0.01 - 0.47)	(0 - 0.22)		(0.01 - 0.42)	(0.01 - 0.5)	(-0.01 - 0.28)	
October 2002[1]	N[2]	258	34		92	138	54	
	Median	0.09	0.06	0.593	0.09	0.08	0.07	0.309
	IQR	(0.02 - 0.34)	(0.03 - 0.23)		(0.02 - 0.28)	(0.02 - 0.4)	(0.03 - 0.17)	
May 2003[1]	N[2]	246	37		90	137	52	
	Median	0.09	0.06	0.19	0.11	0.10	0.06	0.151
	IQR	(0.03 - 0.32)	(0.03 - 0.19)		(0.03 - 0.47)	(0.03 - 0.32)	(0.03 - 0.14)	
October 2003[1]	N[2]	246	38		97	136	52	
	Median	0.10	0.07	0.276	0.10	0.10	0.08	0.365
	IQR	(0.02 - 0.41)	(0.04 - 0.17)		(0.02 - 0.42)	(0.02 - 0.39)	(0.02 - 0.24)	
May 2004[1]	N[2]	231	35		88	124	49	
	Median	0.08	0.05	0.379	0.08	0.07	0.05	0.22
	IQR	(0.02 - 0.25)	(0.02 - 0.15)		(0.03 - 0.34)	(0.02 - 0.24)	(0.02 - 0.14)	
October 2004[1]	N[2]	232	38		92	126	50	
	Median	0.07	0.06	0.721	0.07	0.07	0.03	0.363
	IQR	(0.03 - 0.2)	(0.03 - 0.19)		(0.03 - 0.24)	(0.03 - 0.18)	(0.02 - 0.18)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Wilcoxon rank test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children heterozygous for α-thalassemia (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children homozygous for α-thalassemia (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children heterozygous and homozygous for α-thalassemia (*P*-values not shown).

Table 17 IgG responses to A4 schizont extract in the presence or absence of hemoglobinopathies.

Schizont extract		Sickle trait			Alpha-Thalassemia			
		HbAA	HBAS	*P*-value[3]	Norm	Het	Homo	*P*-value[4]
May 2002[1]	N[2]	257	41		92	153	51	
	Median	0.41	0.25	**0.034**	0.43	0.41	0.33	0.273
	IQR	(0.16 - 0.92)	(0.08 - 0.72)		(0.15 - 0.95)	(0.16 - 0.91)	(0.09 - 0.68)	
October 2002[1]	N[2]	259	34		92	139	54	
	Median	0.13	0.12	0.702	0.13	0.16	0.09	0.15[†]
	IQR	(0.05 - 0.44)	(0.03 - 0.48)		(0.05 - 0.41)	(0.05 - 0.49)	(0.03 - 0.21)	
May 2003[1]	N[2]	249	37		93	137	52	
	Median	0.17	0.13	0.291	0.18	0.19	0.11	0.188
	IQR	(0.06 - 0.54)	(0.04 - 0.42)		(0.06 - 0.48)	(0.05 - 0.57)	(0.05 - 0.31)	
October 2003[1]	N[2]	239	38		95	132	50	
	Median	0.21	0.18	0.218	0.24	0.25	0.14	0.174
	IQR	(0.07 - 0.82)	(0.04 - 0.49)		(0.09 - 0.9)	(0.06 - 0.7)	(0.07 - 0.48)	
May 2004[1]	N[2]	234	35		89	125	49	
	Median	0.09	0.06	0.157	0.10	0.09	0.07	0.865
	IQR	(0.02 - 0.31)	(0.02 - 0.23)		(0.02 - 0.29)	(0.02 - 0.27)	(0.03 - 0.7)	
October 2004[1]	N[2]	232	38		92	126	50	
	Median	0.09	0.10	0.839	0.11	0.10	0.06	0.271
	IQR	(0.04 - 0.25)	(0.05 - 0.22)		(0.03 - 0.35)	(0.04 - 0.25)	(0.04 - 0.17)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Wilcoxon rank test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children heterozygous for α-thalassemia (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children homozygous for α-thalassemia (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children heterozygous and homozygous for α-thalassemia (*P*-values not shown).

Table 18 IgG responses to tetanus toxoid in the presence or absence of hemoglobinopathies.

Tetanus toxoid		Sickle trait			Alpha-Thalassemia			
		HbAA	HBAS	*P*-value[3]	Norm	Het	Homo	*P*-value[4]
May 2002[1]	N[2]	258	41		93	152	52	
	Median	0.54	0.60	0.95	0.60	0.55	0.64	0.543
	IQR	(0.29 - 0.93)	(0.28 - 0.85)		(0.3 - 0.93)	(0.29 - 0.87)	(0.33 - 1.09)	
October 2002[1]	N[2]	258	34		92	138	54	
	Median	0.58	0.60	0.616	0.59	0.58	0.72	0.298
	IQR	(0.33 - 1.03)	(0.37 - 1.13)		(0.35 - 1.1)	(0.3 - 0.95)	(0.36 - 1.27)	
May 2003[1]	N[2]	246	37		90	137	52	
	Median	0.54	0.53	0.69	0.61	0.49	0.86	0.208
	IQR	(0.28 - 1.13)	(0.27 - 0.99)		(0.33 - 1.13)	(0.27 - 1.04)	(0.31 - 1.63)	
October 2003[1]	N[2]	246	38		97	136	52	
	Median	0.48	0.53	0.954	0.55	0.44	0.70	0.1[†]
	IQR	(0.26 - 0.99)	(0.22 - 0.98)		(0.22 - 1.06)	(0.26 - 0.86)	(0.42 - 1.08)	
May 2004[1]	N[2]	231	35		88	124	49	
	Median	0.45	0.53	0.527	0.58	0.44	0.53	0.485
	IQR	(0.26 - 0.91)	(0.37 - 1)		(0.28 - 0.92)	(0.27 - 0.79)	(0.32 - 1.05)	
October 2004[1]	N[2]	232	38		92	126	50	
	Median	0.50	0.58	0.869	0.58	0.46	0.61	0.499
	IQR	(0.29 - 0.99)	(0.28 - 1.03)		(0.3 - 1.05)	(0.28 - 0.94)	(0.33 - 1.04)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Wilcoxon rank test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children heterozygous for α-thalassemia (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children homozygous for α-thalassemia (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children heterozygous and homozygous for α-thalassemia (*P*-values not shown).

Table 19 IgG responses to AMA1(W2mef) in the presence and absence of the sickle trait in different age groups.

AMA1(W2mef)		HbAA[Δ]					HbAS[Δ]				
		0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[3]	0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[4]
May 2002[1]	N[2]	23	109	102	19		5	18	14	4	
	Median	0.04	0.02	0.32	0.57	**<0.001**[*]	0.02	0.01	0.07	0.29	0.258
	IQR	(-0.01 - 0.26)	(-0.02 - 0.17)	(0.03 - 1.63)	(0.13 - 2.1)		(0 - 0.06)	(-0.02 - 0.23)	(0.01 - 0.23)	(0.05 - 1.18)	
October 2002[1]	N[2]	33	94	96	32		3	15	12	4	
	Median	0.66	0.17	0.69	1.64	**<0.001**[*]	1.32	0.17	0.37	0.62	0.434
	IQR	(0.1 - 1.53)	(0.01 - 0.47)	(0.2 - 2.03)	(0.56 - 2.42)		(0.03 - 1.43)	(0.02 - 0.59)	(0.17 - 1)	(0.18 - 1.55)	
May 2003[1]	N[2]	22	88	86	47		3	11	18	4	
	Median	0.01	0.01[†]	0.11[†]	0.81	**<0.001**[*]	0.29	-0.02[†]	0.03[†]	0.13	0.111[*]
	IQR	(-0.03 - 0.09)	(-0.02 - 0.12)	(0.01 - 0.97)	(0.09 - 2.09)		(-0.02 - 0.66)	(-0.04 - 0.01)	(-0.03 - 0.2)	(0.02 - 0.53)	
October 2003[1]	N[2]	13	88	91	52		3	11	18	6	
	Median	0.03	0.02	0.18	0.66	**<0.001**[*]	0.11	0.04	0.1	0.14	0.739
	IQR	(0.01 - 0.08)	(-0.01 - 0.11)	(0.01 - 1.22)	(0.08 - 1.92)		(-0.03 - 0.62)	(0.01 - 0.24)	(0.03 - 0.22)	(0.03 - 0.48)	
May 2004[1]	N[2]	6	79	71	71		2	10	15	8	
	Median	0	0	0.05	0.42	**<0.001**[*]	0.01	0.01	0.05	0.14	0.129
	IQR	(-0.03 - 0.1)	(-0.02 - 0.05)	(0 - 0.35)	(0.06 - 1.37)		(-0.01 - 0.03)	(-0.01 - 0.05)	(0.03 - 0.1)	(0.04 - 0.26)	
October 2004[1]	N[2]	1	64	70	75		-	9	14	12	
	Median	-0.03	-0.01	0.03	0.18	**<0.001**[*]	-	-0.03	0.05	0.09	0.182
	IQR	(-0.03 - -0.03)	(-0.03 - 0.02)	(-0.02 - 0.26)	(0.02 - 0.99)		-	(-0.03 - 0.06)	(0 - 0.38)	(-0.01 - 0.35)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between HbAA children and HbAS children in the same age group (*P*-values not shown).

[Δ] HbAA = normal, HbAS = Sickle trait

Table 20 IgG responses to AMA1(HB3) in the presence and absence of the sickle trait in different age groups.

AMA1(HB3)		HbAA[Δ]					HbAS[Δ]				
		0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[3]	0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[4]
May 2002[1]	N[2]	23	109	102	19		5	18	14	4	
	Median	0.01	0	0.34	0.53	**<0.001**[*]	0.03	-0.01	0.12	0.36	0.135
	IQR	(-0.03 - 0.29)	(-0.04 - 0.21)	(0.06 - 1.55)	(0.18 - 2.14)		(-0.02 - 0.08)	(-0.03 - 0.25)	(0.03 - 0.31)	(0.07 - 1.24)	
October 2002[1]	N[2]	33	94	96	32		3	15	12	4	
	Median	0.12	0.03	0.17	0.49	**<0.001**[*]	0.43	0.05	0.16	0.22	0.27
	IQR	(0.05 - 0.58)	(-0.02 - 0.11)	(0.04 - 0.85)	(0.12 - 1.83)		(0.08 - 0.49)	(0.01 - 0.18)	(0.05 - 0.39)	(-0.02 - 0.83)	
May 2003[1]	N[2]	22	88	86	47		3	11	18	4	
	Median	0.04	0.04	0.17[†]	0.79	**<0.001**[*]	0.47	0	0.06[†]	0.23	0.12
	IQR	(-0.01 - 0.13)	(0 - 0.19)	(0.03 - 1.27)	(0.15 - 2.08)		(0.03 - 0.93)	(-0.02 - 0.06)	(0 - 0.16)	(0.07 - 0.68)	
October 2003[1]	N[2]	13	88	91	52		3	11	18	6	
	Median	-0.02	-0.01	0.09	0.69	**<0.001**[*]	-0.04	0.03	0.11	0.11	0.655
	IQR	(-0.04 - 0.04)	(-0.05 - 0.13)	(-0.04 - 1.24)	(0.05 - 2.02)		(-0.08 - 0.53)	(-0.04 - 0.2)	(-0.03 - 0.2)	(-0.03 - 0.2)	
May 2004[1]	N[2]	6	79	71	71		2	10	15	8	
	Median	-0.01	0	0.07	0.4	**<0.001**[*]	0	0	0.08	0.16	0.192
	IQR	(-0.03 - 0.13)	(-0.02 - 0.08)	(0 - 0.38)	(0.07 - 1.38)		(-0.01 - 0.02)	(-0.02 - 0.03)	(0.01 - 0.17)	(0 - 0.31)	
October 2004[1]	N[2]	1	64	70	75		-	9	14	12	
	Median	-0.08	-0.04	0.02	0.17	**<0.001**[*]	-	-0.05	0.08	0.11	0.348
	IQR	(-0.08 - -0.08)	(-0.06 - 0.01)	(-0.04 - 0.29)	(0 - 0.93)		-	(-0.07 - 0.04)	(-0.03 - 0.38)	(-0.05 - 0.44)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between HbAA children and HbAS children in the same age group (*P*-values not shown).

[Δ] HbAA = normal, HbAS = Sickle trait

Table 21 IgG responses to AMA1(3D7) in the presence and absence of the sickle trait in different age groups.

AMA1(3D7)		HbAA[Δ]					HbAS[Δ]				
		0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[3]	0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[4]
May 2002[1]	N[2]	23	109	102	19		5	18	14	4	
	Median	0.03	0.02	0.24	0.35	**<0.001**[*]	0.01	0.01	0.08	0.27	0.104
	IQR	(0 - 0.25)	(-0.01 - 0.12)	(0.04 - 1.28)	(0.07 - 1.88)		(0 - 0.04)	(-0.01 - 0.15)	(0.02 - 0.18)	(0.06 - 0.97)	
October 2002[1]	N[2]	33	94	96	32		3	15	12	4	
	Median	0.07	0	0.13	0.43	**<0.001**[*]	0.17	0.03	0.08	0.19	0.335
	IQR	(0 - 0.23)	(-0.02 - 0.07)	(0.02 - 0.57)	(0.06 - 1.61)		(0.01 - 0.25)	(-0.03 - 0.1)	(0 - 0.21)	(0.02 - 0.64)	
May 2003[1]	N[2]	22	88	86	47		3	11	18	4	
	Median	0.02	0.03	0.1[†]	0.68	**<0.001**[*]	0.65	0	0.04[†]	0.17	0.149
	IQR	(0 - 0.14)	(-0.01 - 0.1)	(0.02 - 0.84)	(0.1 - 2.06)		(0.01 - 1.16)	(-0.02 - 0.03)	(0 - 0.22)	(0.04 - 0.46)	
October 2003[1]	N[2]	13	88	91	52		3	11	18	6	
	Median	0	0.01	0.1	0.48	**<0.001**[*]	0.03	0.04	0.1	0.14	0.479
	IQR	(-0.02 - 0.08)	(-0.02 - 0.11)	(0 - 0.65)	(0.06 - 1.89)		(-0.04 - 0.57)	(-0.02 - 0.2)	(0.03 - 0.18)	(0.04 - 0.19)	
May 2004[1]	N[2]	6	79	71	71		2	10	15	8	
	Median	0.01	0	0.04	0.35	**<0.001**[*]	0.01	0.01	0.06	0.17	0.339
	IQR	(-0.03 - 0.16)	(-0.02 - 0.06)	(-0.01 - 0.29)	(0.05 - 1.16)		(-0.01 - 0.04)	(-0.02 - 0.05)	(0.01 - 0.09)	(-0.01 - 0.23)	
October 2004[1]	N[2]	1	64	70	75		-	9	14	12	
	Median	-0.02	0	0.02	0.14	**<0.001**[*]	-	-0.02	0.06	0.06	0.377
	IQR	(-0.02 - -0.02)	(-0.03 - 0.02)	(-0.01 - 0.21)	(0.02 - 0.76)		-	(-0.02 - 0.11)	(0 - 0.24)	(-0.01 - 0.24)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between HbAA children and HbAS children in the same age group (*P*-values not shown).

[Δ] HbAA = normal, HbAS = Sickle trait

Table 22 IgG responses to MSP2(3D7) in the presence and absence of the sickle trait in different age groups.

MSP2(3D7)		HbAA[Δ]					HbAS[Δ]				
		0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[3]	0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[4]
May 2002[1]	N[2]	23	109	102	19		5	18	14	4	
	Median	0.05	0.14	0.35	0.57	**<0.001**[*]	0.03	0.12	0.23	0.4	0.069
	IQR	(0.02 - 0.34)	(0.04 - 0.41)	(0.12 - 1.08)	(0.13 - 1.29)		(0.01 - 0.06)	(0.01 - 0.33)	(0.1 - 1.29)	(0.04 - 1.48)	
October 2002[1]	N[2]	33	94	96	32		3	15	12	4	
	Median	0.03	0.04	0.14	0.14	**<0.001**[*]	0.1	0.06	0.14	0.07	0.295
	IQR	(0.01 - 0.09)	(0.02 - 0.08)	(0.06 - 0.39)	(0.07 - 0.47)		(0.08 - 0.32)	(0.03 - 0.1)	(0.07 - 0.19)	(0.02 - 0.88)	
May 2003[1]	N[2]	22	88	86	47		3	11	18	4	
	Median	0.08	0.04	0.13	0.18	**<0.001**[*]	0.12	0.06	0.11	0.05	0.427
	IQR	(0.01 - 0.23)	(0.01 - 0.1)	(0.05 - 0.44)	(0.09 - 0.72)		(0 - 0.27)	(0.02 - 0.07)	(0.04 - 0.21)	(0.01 - 0.67)	
October 2003[1]	N[2]	13	88	91	52		3	11	18	6	
	Median	0.07	0.09	0.13	0.23	**<0.001**[*]	0.06	0.05	0.13	0.23	**0.023**[*]
	IQR	(0.02 - 0.12)	(0.02 - 0.31)	(0.04 - 0.43)	(0.08 - 0.6)		(0.05 - 0.06)	(0.01 - 0.15)	(0.08 - 0.4)	(0.06 - 0.38)	
May 2004[1]	N[2]	6	79	71	71		2	10	15	8	
	Median	0	0.04	0.08	0.24	**<0.001**[*]	0.03	0.03	0.12	0.07	**0.052**
	IQR	(-0.01 - 0.01)	(0 - 0.11)	(0.03 - 0.24)	(0.07 - 0.6)		(-0.01 - 0.08)	(0.01 - 0.04)	(0.07 - 0.38)	(0.03 - 0.38)	
October 2004[1]	N[2]	1	64	70	75		-	9	14	12	
	Median	0.02	0.04	0.07	0.19	**<0.001**[*]	-	0.05	0.06	0.11	0.702
	IQR	(0.02 - 0.02)	(0.01 - 0.07)	(0.02 - 0.13)	(0.05 - 0.51)		-	(0.02 - 0.09)	(0.04 - 0.16)	(0.04 - 0.35)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between HbAA children and HbAS children in the same age group (*P*-values not shown).

[Δ] HbAA = normal, HbAS = Sickle trait

Table 23 IgG responses to MSP2(FC27) in the presence and absence of the sickle trait in different age groups.

MSP2(FC27)		HbAA[Δ]					HbAS[Δ]				
		0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[3]	0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[4]
May 2002[1]	N[2]	23	109	102	19		5	18	14	4	
	Median	0.01	0.04	0.2[†]	0.31	**<0.001**[*]	-0.01	0.02	0.01[†]	0.37	**0.049**
	IQR	(-0.01 - 0.22)	(0 - 0.23)	(0.03 - 0.51)	(0.13 - 1.21)		(-0.02 - 0)	(-0.01 - 0.35)	(0.01 - 0.12)	(0.2 - 0.55)	
October 2002[1]	N[2]	33	94	96	32		3	15	12	4	
	Median	0.07	0.03	0.16	0.31	**<0.001**[*]	0.06	0.06	0.06	0.13	0.751
	IQR	(0.02 - 0.12)	(0 - 0.11)	(0.03 - 0.48)	(0.1 - 0.89)		(0.06 - 0.1)	(0 - 0.23)	(0.03 - 0.4)	(0.04 - 0.37)	
May 2003[1]	N[2]	22	88	86	47		3	11	18	4	
	Median	0.05	0.06	0.13	0.23	**<0.001**[*]	0.04	0.07	0.08	0.23	0.881
	IQR	(0.02 - 0.08)	(0.02 - 0.17)	(0.04 - 0.56)	(0.07 - 0.96)		(0 - 0.14)	(0.03 - 0.19)	(0.03 - 0.21)	(0.01 - 0.73)	
October 2003[1]	N[2]	13	88	91	52		3	11	18	6	
	Median	0.04	0.06	0.12	0.22	**<0.001**[*]	0.03	0.05	0.09	0.11	0.536
	IQR	(0.01 - 0.06)	(0.01 - 0.23)	(0.02 - 0.5)	(0.09 - 1.22)		(0 - 0.16)	(0.04 - 0.12)	(0.04 - 0.22)	(0.05 - 0.38)	
May 2004[1]	N[2]	6	79	71	71		2	10	15	8	
	Median	0	0.04	0.07	0.18	**<0.001**[*]	0.03	0.03	0.12	0.1	0.063
	IQR	(-0.01 - 0.02)	(0.01 - 0.14)	(0.02 - 0.22)	(0.07 - 0.58)		(-0.01 - 0.08)	(0.02 - 0.05)	(0.04 - 0.22)	(0.01 - 0.39)	
October 2004[1]	N[2]	1	64	70	75		-	9	14	12	
	Median	0	0.04	0.07	0.13	**<0.001**[*]	-	0.03	0.04	0.13	0.246
	IQR	(0 - 0)	(0.01 - 0.1)	(0.03 - 0.19)	(0.04 - 0.34)		-	(0.01 - 0.08)	(0.03 - 0.16)	(0.03 - 0.28)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between HbAA children and HbAS children in the same age group (*P*-values not shown).

[Δ] HbAA = normal, HbAS = Sickle trait

Table 24 IgG responses to A4 schizont extract in the presence and absence of the sickle trait in different age groups.

Schizont extract		HbAA[Δ]					HbAS[Δ]				
		0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[3]	0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[4]
May 2002[1]	N[2]	23	108	102	19		5	18	14	4	
	Median	0.18	0.23	0.69[†]	0.93	**<0.001**[*]	0.08	0.14	0.25[†]	0.68	0.095
	IQR	(0.07 - 0.43)	(0.1 - 0.54)	(0.36 - 1.1)	(0.34 - 1.23)		(0.07 - 0.08)	(0.06 - 0.98)	(0.17 - 0.44)	(0.59 - 0.87)	
October 2002[1]	N[2]	33	95	96	32		3	15	12	4	
	Median	0.07	0.07	0.27	0.34	**<0.001**[*]	0.21	0.03	0.17	0.15	0.383
	IQR	(0.02 - 0.19)	(0.02 - 0.15)	(0.09 - 0.57)	(0.13 - 0.92)		(0 - 0.54)	(0.01 - 0.31)	(0.08 - 0.53)	(0.07 - 0.36)	
May 2003[1]	N[2]	22	89	87	48		3	11	18	4	
	Median	0.03	0.1	0.21	0.56	**<0.001**[*]	0.03	0.13	0.14	0.4	0.595
	IQR	(0.02 - 0.14)	(0.05 - 0.22)	(0.06 - 0.63)	(0.23 - 1.02)		(0 - 0.33)	(0.01 - 0.55)	(0.06 - 0.42)	(0.13 - 0.7)	
October 2003[1]	N[2]	13	85	88	51		3	11	18	6	
	Median	0.1	0.11	0.27	0.58	**<0.001**[*]	0.07	0.1	0.29	0.39	0.143
	IQR	(0.03 - 0.17)	(0.04 - 0.36)	(0.1 - 0.95)	(0.28 - 1.05)		(0.02 - 0.33)	(0.02 - 0.24)	(0.11 - 0.71)	(0.04 - 0.7)	
May 2004[1]	N[2]	6	80	72	71		2	10	15	8	
	Median	0.18	0.19	0.07	0.04	**<0.001**[*]	0.15	0.19	0.03	0.02	**0.011**[*]
	IQR	(0.12 - 1.03)	(0.08 - 0.4)	(0.02 - 0.29)	(0.01 - 0.11)		(0.06 - 0.25)	(0.1 - 0.29)	(0.02 - 0.07)	(0 - 0.05)	
October 2004[1]	N[2]	1	64	70	75		-	9	14	12	
	Median	0.01	0.03	0.09	0.2	**<0.001**[*]	-	0.02	0.07	0.14	0.069[*]
	IQR	(0.01 - 0.01)	(0.01 - 0.07)	(0.04 - 0.2)	(0.1 - 0.49)		-	(0.01 - 0.1)	(0.06 - 0.15)	(0.1 - 0.22)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between HbAA children and HbAS children in the same age group (*P*-values not shown).

[Δ] HbAA = normal, HbAS = Sickle trait

Table 25 IgG responses to tetanus toxoid in the presence and absence of the sickle trait in different age groups.

Tetanus toxoid		HbAA[Δ]					HbAS[Δ]				
		0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[3]	0 years	1 – 3 years	4 – 6 years	7 – 10 years	*P*-value[4]
May 2002[1]	N[2]	23	109	102	19		5	18	14	4	
	Median	0.85	0.68	0.43	0.36	**0.001**[*]	1.37	0.61	0.42	0.41	0.182
	IQR	(0.16 - 1.43)	(0.39 - 1.06)	(0.28 - 0.77)	(0.14 - 0.58)		(0.67 - 1.55)	(0.29 - 0.93)	(0.28 - 0.66)	(0.26 - 0.75)	
October 2002[1]	N[2]	33	94	96	32		3	15	12	4	
	Median	1.1	0.75	0.48	0.44	**0.003**[*]	1.13	0.64	0.52	0.34	0.305
	IQR	(0.27 - 2.02)	(0.39 - 1.11)	(0.33 - 0.74)	(0.27 - 0.62)		(0.8 - 1.88)	(0.5 - 1.22)	(0.31 - 1.03)	(0.25 - 0.87)	
May 2003[1]	N[2]	22	88	86	47		3	11	18	4	
	Median	1.39	0.57	0.47	0.36	**<0.001**	0.86	0.73	0.49	0.39	0.362
	IQR	(1.09 - 1.97)	(0.28 - 1.02)	(0.27 - 0.92)	(0.21 - 2.11)		(0.47 - 2.04)	(0.29 - 1.25)	(0.18 - 0.6)	(0.24 - 0.71)	
October 2003[1]	N[2]	13	88	91	52		3	11	18	6	
	Median	0.95	0.61	0.42	0.34	0.059	1	0.72	0.59	0.24	0.079
	IQR	(0.38 - 1.93)	(0.38 - 1)	(0.24 - 0.79)	(0.22 - 1.03)		(0.54 - 2.27)	(0.2 - 1.28)	(0.31 - 0.87)	(0.18 - 0.3)	
May 2004[1]	N[2]	6	79	71	71		2	10	15	8	
	Median	0.67	0.6	0.44	0.36	0.475	0.57	0.9	0.58	0.27	0.096[*]
	IQR	(0.34 - 2.24)	(0.28 - 0.92)	(0.26 - 0.88)	(0.24 - 1.33)		(0.46 - 0.68)	(0.43 - 1.11)	(0.37 - 0.96)	(0.2 - 0.55)	
October 2004[1]	N[2]	1	64	70	75		-	9	14	12	
	Median	0.19	0.64	0.57	0.42	0.652	-	0.88	0.59	0.31	0.125[*]
	IQR	(0.19 - 0.19)	(0.3 - 1.09)	(0.29 - 0.98)	(0.3 - 1.48)		-	(0.49 - 1.24)	(0.22 - 1.03)	(0.21 - 0.63)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between HbAA children and HbAS children in the same age group (*P*-values not shown).

[Δ] HbAA = normal, HbAS = Sickle trait

Table 26 IgG responses to AMA1(W2mef) in the presence and absence of alpha-thalassemia in different age groups.

AMA1(W2mef)		Normal					Alpha-Thalassemia HETEROZYGOUS					Alpha-Thalassemia HOMOZYGOUS				
		0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 – 3 years	4 – 6 years	7 – 10 years	P^3
May 2002[1]	N[2]	10	44	32	5		14	55	64	16		5	25	21	1	
	Median	0.06	0.04	0.3	0.37	**0.01**	0.04	0.01	0.25	0.47	S[*]	0.02	0.04	0.1	1.67	**0.03**
	IQR	(-0.02 - 0.23)	(-0.03 - 0.6)	(0.02 - 1.83)	(0.36 - 0.57)		(0 - 0.18)	(-0.02 - 0.13)	(0.04 - 1)	(0.09 - 1.88)		(0 - 0.02)	(-0.01 - 0.1)	(0.04 - 0.42)	(1.67 - 1.67)	
October 2002[1]	N[2]	15	39	27	10		14	40	62	20		3	27	18	6	
	p50[Δ]	0.36	0.19	0.73	1.89	**0.003**[*]	0.62	0.17	0.9	1.57	S[*]	2.14	0.08	0.38	1.05	**0.039**
	IQR	(0.03 - 1.29)	(0.03 - 0.48)	(0.12 - 2)	(0.68 - 2.47)		(0.13 - 1.32)	(0 - 0.44)	(0.25 - 2.1)	(0.55 - 2.37)		(0.16 - 2.16)	(0.03 - 0.37)	(0.13 - 0.84)	(0.09 - 2.29)	
May 2003[1]	N[2]	7	35	32	13		10	39	57	30		6	24	15	7	
	Median	-0.01	0.01	0.11	0.19	**0.005**[*]	0.06	0.01	0.07	0.75	S[*]	0.01	-0.01	0.05	0.29	**0.021**[*]
	IQR	(-0.05 - 0.13)	(-0.01 - 0.13)	(0.01 - 0.57)	(0.06 - 2.2)		(0 - 0.17)	(-0.03 - 0.16)	(0 - 0.84)	(0.1 - 1.6)		(-0.04 - 0.02)	(-0.04 - 0.05)	(-0.02 - 0.87)	(0.03 - 1.86)	
October 2003[1]	N[2]	7	35	36	17		9	40	54	33		4	22	19	7	
	Median	0.14	0.04	0.14	0.38	**0.024**[*]	0.08	0.01	0.17	0.51	S[*]	0.06	0.01	0.07	0.63	**0.043***
	IQR	(0.03 - 0.28)	(0 - 0.37)	(0.02 - 1.65)	(0.07 - 2.15)		(0.02 - 0.24)	(-0.01 - 0.07)	(0.02 - 0.65)	(0.07 - 1.61)		(0 - 0.11)	(-0.01 - 0.06)	(0.01 - 0.25)	(0.1 - 1.67)	
May 2004[1]	N[2]	3	34	28	22		2	37	38	45		1	19	18	11	
	Median	-0.03	0.03[†‡¢]	0.06	0.93	S[*]	-0.01	0[†‡]	0.05	0.38	S[*]	-0.01	-0.01[†¢]	0.04	0.08	**0.014**[*]
	IQR	(-0.03 - 0.01)	(-0.01 - 0.21)	(0.02 - 0.37)	(0.1 - 1.68)		(-0.01 - 0)	(-0.02 - 0.01)	(0 - 0.34)	(0.06 - 1.1)		(-0.01 - -0.01)	(-0.02 - 0.02)	(-0.01 - 0.2)	(0 - 0.31)	
October 2004[1]	N[2]	-	26	31	26		-	30	39	47		-	17	15	14	
	Median	-	-0.01	0.04	0.18	S[*]	-	-0.02	0.03	0.32[‡]	S[*]	-	-0.01	0.05	0.06[‡]	0.147
	IQR	-	(-0.03 - 0.03)	(-0.01 - 0.38)	(0.02 - 1.24)		-	(-0.03 - 0.02)	(-0.03 - 0.17)	(0.03 - 0.88)		-	(-0.03 - 0.01)	(0 - 0.26)	(-0.03 - 0.15)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type, S = $P < 0.001$).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Kruskal Wallis) in median IgG levels between normal, α-thal heterozygous, and α-thal homozygous children in the same age group (*P*-values not shown).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal and α-thal heterozygous children in the same age group (*P*-values not shown).

[¢] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal and α-thal homozygous children in the same age group (*P*-values not shown).

[ξ] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between α-thal heterozygous and α-thal homozygous children in the same age group (*P*-values not shown).

Table 27 IgG responses to AMA1(HB3) in the presence and absence of alpha-thalassemia in different age groups.

AMA1(HB3)		Normal					Alpha-Thalassemia HETEROZYGOUS					Alpha-Thalassemia HOMOZYGOUS				
		0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 – 3 years	4 – 6 years	7 – 10 years	P^3
May 2002[1]	N[2]	10	44	32	5		14	55	64	16		5	25	21	1	
	Median	0.01	-0.01	0.43	0.4	**0.001**[*]	0.02	0.01	0.3	0.45	S[*]	0.03	-0.02	0.16	1.98	**0.003**
	IQR	(-0.05 - 0.14)	(-0.04 - 0.6)	(0.03 - 1.82)	(0.24 - 0.84)		(-0.01 - 0.14)	(-0.03 - 0.21)	(0.05 - 1.24)	(0.1 - 1.8)		(-0.02 - 0.03)	(-0.04 - 0.1)	(0.06 - 0.53)	(1.98 - 1.98)	
October 2002[1]	N[2]	15	39	27	10		14	40	62	20		3	27	18	6	
	p50[Δ]	0.08	0.07[†€]	0.23	0.74	**0.041**[*]	0.18	0.04[†]	0.17	0.44	S[*]	0.67	-0.01[†€]	0.15	0.21	**0.004**[*]
	IQR	(0.02 - 0.39)	(0 - 0.37)	(0.01 - 1.15)	(0.15 - 2.12)		(0.05 - 0.46)	(-0.03 - 0.14)	(0.06 - 0.88)	(0.1 - 1.5)		(0.02 - 1.12)	(-0.04 - 0.05)	(0.03 - 0.37)	(0.09 - 1.03)	
May 2003[1]	N[2]	7	35	32	13		10	39	57	30		6	24	15	7	
	Median	0.01	0.05	0.18	0.7	**0.007**[*]	0.1[Σ]	0.03	0.12	0.64	S[*]	0[Σ]	0.01	0.11	0.26	**0.002**[*]
	IQR	(-0.02 - 0.21)	(0 - 0.2)	(0.04 - 0.57)	(0.13 - 2.11)		(0.04 - 0.26)	(-0.01 - 0.2)	(0.03 - 0.86)	(0.19 - 1.77)		(-0.01 - 0.01)	(-0.01 - 0.07)	(0.02 - 1.27)	(0.09 - 1.95)	
October 2003[1]	N[2]	7	35	36	17		9	40	54	33		4	22	19	7	
	Median	0.16	0.02	0.18	0.91	**0.007**[*]	0.01	-0.02	0.1	0.56	S[*]	-0.03	-0.02	0.01	0.88	0.08[*]
	IQR	(-0.01 - 0.31)	(-0.05 - 0.22)	(0 - 1.6)	(0.03 - 2.1)		(-0.04 - 0.28)	(-0.05 - 0.06)	(-0.03 - 0.77)	(0.05 - 1.33)		(-0.04 - 0.03)	(-0.05 - 0.03)	(-0.04 - 0.18)	(0.03 - 1.84)	
May 2004[1]	N[2]	3	34	28	22		2	37	38	45		1	19	18	11	
	Median	-0.03	0.02	0.09	0.95	S[*]	0.03	-0.01	0.05	0.33	S[*]	-0.01	0	0.06	0.08	**0.046**[*]
	IQR	(-0.03 - 0)	(-0.02 - 0.22)	(0.02 - 0.36)	(0.13 - 1.75)		(0.02 - 0.04)	(-0.03 - 0.04)	(0 - 0.41)	(0.05 - 0.93)		(-0.01 - -0.01)	(-0.03 - 0.04)	(0 - 0.21)	(0 - 0.38)	
October 2004[1]	N[2]	-	26	31	26		-	30	39	47		-	17	15	14	
	Median	-	-0.05	0.08	0.24	S[*]	-	-0.05	0.02	0.17	S[*]	-	-0.04	0	-0.01	0.282
	IQR	-	(-0.07 - 0.01)	(-0.02 - 0.38)	(0.03 - 1.47)		-	(-0.07 - 0.01)	(-0.06 - 0.21)	(0 - 0.79)		-	(-0.05 - -0.01)	(-0.04 - 0.36)	(-0.05 - 0.24)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type, S = *P* < 0.001).

[*] Significant difference (*P* ≤ 0.05 Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

[†] Significant difference (*P* ≤ 0.05 Kruskal Wallis) in median IgG levels between normal, α-thal heterozygous, and α-thal homozygous children in the same age group (*P*-values not shown).

[Δ] Significant difference (*P* ≤ 0.05 Wilcoxon rank test) in median IgG levels between normal and α-thal heterozygous children in the same age group (*P*-values not shown).

[€] Significant difference (*P* ≤ 0.05 Wilcoxon rank test) in median IgG levels between normal and α-thal homozygous children in the same age group (*P*-values not shown).

[Σ] Significant difference (*P* ≤ 0.05 Wilcoxon rank test) in median IgG levels between α-thal heterozygous and α-thal homozygous children in the same age group (*P*-values not shown).

Table 28 IgG responses to AMA1(3D7) in the presence and absence of alpha-thalassemia in different age groups.

AMA1(3D7)		Normal					Alpha-Thalassemia HETEROZYGOUS					Alpha-Thalassemia HOMOZYGOUS				
		0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 – 3 years	4 – 6 years	7 – 10 years	P^3
May 2002[1]	N[2]	10	44	32	5		14	55	64	16		5	25	21	1	
	Median	0.02	0.02	0.29	0.09	**0.011**	0.04	0.02	0.19	0.41	S*	0.03	0.02	0.11	1.88	**0.01**
	IQR	(-0.02 - 0.08)	(-0.01 - 0.47)	(0.03 - 1.65)	(0.07 - 0.27)		(0 - 0.12)	(-0.01 - 0.12)	(0.05 - 0.76)	(0.07 - 1.62)		(0.01 - 0.04)	(-0.01 - 0.07)	(0.03 - 0.37)	(1.88 - 1.88)	
October 2002[1]	N[2]	15	39	27	10		14	40	62	20		3	27	18	6	
	p50[Δ]	0.02[†]	0.02[‡]	0.12	0.41	**0.008***	0.06[†]	-0.01[‡ξ]	0.15	0.43	S*	0.33[†ξ]	0	0.06	0.09	**0.011***
	IQR	(-0.01 - 0.2)	(-0.01 - 0.12)	(0 - 0.57)	(0.05 - 2)		(0 - 0.25)	(-0.03 - 0.05)	(0.04 - 0.6)	(0.18 - 1.53)		(0.01 - 0.97)	(-0.02 - 0.05)	(-0.01 - 0.22)	(0.02 - 0.56)	
May 2003[1]	N[2]	7	35	32	13		10	39	57	30		6	24	15	7	
	Median	0.01	0.03	0.16	0.64	**0.003***	0.08	0.02	0.1	0.67	S*	0	0	0.08	0.28	**0.005***
	IQR	(-0.02 - 0.57)	(-0.01 - 0.15)	(0.01 - 0.47)	(0.07 - 2.1)		(0.02 - 0.16)	(-0.01 - 0.08)	(0.03 - 0.52)	(0.13 - 1.68)		(-0.02 - 0.02)	(-0.02 - 0.06)	(0.01 - 1.03)	(0.07 - 1.82)	
October 2003[1]	N[2]	7	35	36	17		9	40	54	33		4	22	19	7	
	Median	0.05	0.02	0.19	0.74	**0.013***	0.08	0	0.11	0.38	S*	0.01	0	0.07	0.41	**0.052***
	IQR	(0.01 - 0.16)	(-0.01 - 0.24)	(0 - 1.57)	(0.03 - 1.95)		(0 - 0.24)	(-0.02 - 0.08)	(0.01 - 0.45)	(0.06 - 1.72)		(-0.02 - 0.06)	(-0.01 - 0.05)	(-0.01 - 0.22)	(0.13 - 1.64)	
May 2004[1]	N[2]	3	34	28	22		2	37	38	45		1	19	18	11	
	Median	-0.03	0.04[†ξ]	0.06	0.71	**0.001***	0.01	0[†]	0.04	0.29	S*	-0.01	-0.01[†ξ]	0.04	0.1	**0.007***
	IQR	(-0.03 - 0.02)	(-0.01 - 0.19)	(0 - 0.26)	(0.12 - 1.52)		(-0.01 - 0.03)	(-0.02 - 0.03)	(0.01 - 0.16)	(0.04 - 1)		(-0.01 - -0.01)	(-0.02 - 0)	(-0.01 - 0.12)	(0 - 0.27)	
October 2004[1]	N[2]	-	26	31	26		-	30	39	47		-	17	15	14	
	Median	-	0	0.07	0.17	S*	-	-0.02	0.03	0.17	S*	-	0	0.01	0.06	0.101*
	IQR	-	(-0.03 - 0.02)	(-0.01 - 0.29)	(0.01 - 0.8)		-	(-0.03 - 0.02)	(-0.02 - 0.1)	(0.02 - 0.76)		-	(-0.02 - 0.02)	(-0.02 - 0.22)	(0 - 0.15)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type, S = $P < 0.001$).

* Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

† Significant difference ($P \leq 0.05$ Kruskal Wallis) in median IgG levels between normal, α-thal heterozygous, and α-thal homozygous children in the same age group (*P*-values not shown).

‡ Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal and α-thal heterozygous children in the same age group (*P*-values not shown).

ξ Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal and α-thal homozygous children in the same age group (*P*-values not shown).

Σ Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between α-thal heterozygous and α-thal homozygous children in the same age group (*P*-values not shown).

Table 29 IgG responses to MSP2(3D7) in the presence and absence of alpha-thalassemia in different age groups.

MSP2(3D7)		Normal					Alpha-Thalassemia HETEROZYGOUS					Alpha-Thalassemia HOMOZYGOUS				
		0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 – 3 years	4 – 6 years	7 – 10 years	P^3
May 2002[1]	N[2]	10	44	32	5		14	55	64	16		5	25	21	1	
	Median	0.04	0.24[ε]	0.29	0.32	**0.013**	0.06[Σ]	0.14	0.35	0.59	**0.001**[*]	0.03[Σ]	0.11[ε]	0.3	0.11	**0.009**
	IQR	(0.02 - 0.13)	(0.06 - 0.65)	(0.16 - 1.12)	(0.29 - 2.13)		(0.03 - 0.34)	(0.02 - 0.42)	(0.1 - 1.29)	(0.12 - 1)		(0.01 - 0.03)	(0.03 - 0.2)	(0.1 - 0.79)	(0.11 - 0.11)	
October 2002[1]	N[2]	15	39	27	10		14	40	62	20		3	27	18	6	
	p50[δ]	0.05	0.05	0.14	0.23	S[*]	0.07	0.04	0.14	0.12	S[*]	0.04	0.05	0.13	0.16	0.066
	IQR	(0.02 - 0.08)	(0.02 - 0.11)	(0.08 - 0.56)	(0.08 - 1.01)		(0.03 - 0.24)	(0.02 - 0.08)	(0.05 - 0.4)	(0.07 - 0.45)		(0.01 - 0.15)	(0.02 - 0.09)	(0.07 - 0.21)	(0.06 - 0.22)	
May 2003[1]	N[2]	7	35	32	13		10	39	57	30		6	24	15	7	
	Median	0.09	0.03	0.23	0.2	**0.001**[*]	0.08	0.03	0.1	0.16	S[*]	0.16	0.05	0.11	0.13	0.158
	IQR	(0.02 - 0.23)	(0 - 0.12)	(0.08 - 0.93)	(0.1 - 0.32)		(0 - 0.11)	(0.02 - 0.09)	(0.04 - 0.31)	(0.07 - 0.4)		(0.06 - 0.33)	(0.01 - 0.1)	(0.04 - 0.46)	(0.02 - 1.02)	
October 2003[1]	N[2]	7	35	36	17		9	40	54	33		4	22	19	7	
	Median	0.12	0.13	0.15	0.21	0.177[*]	0.08	0.05	0.13	0.21	**0.002**[*]	0.05	0.05	0.1	0.33	0.1
	IQR	(0.04 - 0.19)	(0.03 - 0.34)	(0.06 - 0.45)	(0.12 - 0.63)		(0.05 - 0.19)	(0 - 0.15)	(0.05 - 0.45)	(0.07 - 0.54)		(0.02 - 0.06)	(0.02 - 0.33)	(0.05 - 0.19)	(0.14 - 0.49)	
May 2004[1]	N[2]	3	34	28	22		2	37	38	45		1	19	18	11	
	Median	0	0.04	0.1	0.18	**0.002**[*]	0.03	0.01	0.08	0.25	S[*]	-0.01	0.05	0.08	0.17	0.06[*]
	IQR	(-0.01 - 0.01)	(0 - 0.16)	(0.05 - 0.34)	(0.05 - 0.98)		(0.01 - 0.05)	(0 - 0.05)	(0.04 - 0.24)	(0.06 - 0.6)		(-0.01 - -0.01)	(0.02 - 0.1)	(0.04 - 0.21)	(0.07 - 0.29)	
October 2004[1]	N[2]	-	26	31	26		-	30	39	47		-	17	15	14	
	Median	-	0.05	0.09	0.34	S[*]	-	0.02[Σ]	0.07	0.17	S[*]	-	0.04[Σ]	0.05	0.09	0.722
	IQR	-	(0.01 - 0.07)	(0.04 - 0.19)	(0.06 - 1.03)		-	(0.01 - 0.08)	(0.01 - 0.13)	(0.08 - 0.41)		-	(0.03 - 0.08)	(0.02 - 0.16)	(0.01 - 0.44)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type, S = *P* < 0.001).

[*] Significant difference (*P* ≤ 0.05 Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

[†] Significant difference (*P* ≤ 0.05 Kruskal Wallis) in median IgG levels between normal, α-thal heterozygous, and α-thal homozygous children in the same age group (*P*-values not shown).

[‡] Significant difference (*P* ≤ 0.05 Wilcoxon rank test) in median IgG levels between normal and α-thal heterozygous children in the same age group (*P*-values not shown).

[ε] Significant difference (*P* ≤ 0.05 Wilcoxon rank test) in median IgG levels between normal and α-thal homozygous children in the same age group (*P*-values not shown).

[Σ] Significant difference (*P* ≤ 0.05 Wilcoxon rank test) in median IgG levels between α-thal heterozygous and α-thal homozygous children in the same age group (*P*-values not shown).

Table 30 IgG responses to MSP2(FC27) in the presence and absence of alpha-thalassemia in different age groups.

MSP2(FC27)		Normal					Alpha-Thalassemia HETEROZYGOUS					Alpha-Thalassemia HOMOZYGOUS				
		0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 – 3 years	4 – 6 years	7 – 10 years	P^3
May 2002[1]	N[2]	10	44	32	5		14	55	64	16		5	25	21	1	
	Median	0.01	0.14	0.23	0.16	**0.026**	0.07	0.03	0.12	0.38	**0.002**[*]	0	0.03	0.12	0.32	0.162
	IQR	(-0.02 - 0.1)	(0.01 - 0.65)	(0.03 - 0.45)	(0.13 - 0.58)		(-0.01 - 0.51)	(-0.01 - 0.19)	(0.02 - 0.5)	(0.15 - 1.58)		(-0.01 - 0.01)	(-0.01 - 0.23)	(0.02 - 0.32)	(0.32 - 0.32)	
October 2002[1]	N[2]	15	39	27	10		14	40	62	20		3	27	18	6	
	p50[Δ]	0.06	0.06	0.17	0.36	**0.019**[*]	0.05	0.03	0.13	0.35	**S**[*]	0.12	0.03	0.2	0.12	**0.018**
	IQR	(0.01 - 0.17)	(0.01 - 0.25)	(0.03 - 0.44)	(0.12 - 0.83)		(0.02 - 0.1)	(0 - 0.11)	(0.03 - 0.55)	(0.1 - 1.38)		(0 - 0.17)	(0 - 0.1)	(0.05 - 0.35)	(0.06 - 0.17)	
May 2003[1]	N[2]	7	35	32	13		10	39	57	30		6	24	15	7	
	Median	0.05	0.04	0.17	0.37	**0.008**[*]	0.06	0.07	0.11	0.22	**0.001**[*]	0.06	0.06	0.07	0.18	0.143[*]
	IQR	(0.02 - 0.15)	(0.01 - 0.27)	(0.07 - 0.72)	(0.11 - 1.09)		(0 - 0.08)	(0.02 - 0.19)	(0.04 - 0.36)	(0.07 - 0.96)		(0.04 - 0.09)	(0.02 - 0.08)	(0.03 - 0.18)	(0.04 - 0.77)	
October 2003[1]	N[2]	7	35	36	17		9	40	54	33		4	22	19	7	
	Median	0.04	0.06	0.11	0.38	**0.008**[*]	0.06	0.05	0.14	0.12	**0.005**[*]	0.03	0.05	0.08	0.28	0.087[*]
	IQR	(0.01 - 0.1)	(0.01 - 0.31)	(0.05 - 0.51)	(0.13 - 1.4)		(0.01 - 0.16)	(0.01 - 0.12)	(0.02 - 0.5)	(0.09 - 1.18)		(0.02 - 0.05)	(0.02 - 0.18)	(0.02 - 0.35)	(0.13 - 0.38)	
May 2004[1]	N[2]	3	34	28	22		2	37	38	45		1	19	18	11	
	Median	-0.01	0.05	0.1	0.27	**0.003**[*]	0.03	0.03	0.07	0.14	**0.001**[*]	-0.01	0.05	0.05	0.09	0.173
	IQR	(-0.01 - 0)	(0.02 - 0.14)	(0.03 - 0.28)	(0.07 - 0.58)		(0.02 - 0.04)	(0.01 - 0.11)	(0.02 - 0.22)	(0.07 - 0.72)		(-0.01 - -0.01)	(0.01 - 0.1)	(0.03 - 0.15)	(0.03 - 0.25)	
October 2004[1]	N[2]	-	26	31	26		-	30	39	47		-	17	15	14	
	Median	-	0.04	0.06	0.17[£]	**0.013**[*]	-	0.04	0.05	0.13	**S**[*]	-	0.03	0.06	0.06[£]	0.84
	IQR	-	(0.01 - 0.13)	(0.02 - 0.19)	(0.04 - 0.47)		-	(0 - 0.07)	(0.02 - 0.14)	(0.04 - 0.35)		-	(0.02 - 0.18)	(0.03 - 0.21)	(0.02 - 0.2)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type, S = $P < 0.001$).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Kruskal Wallis) in median IgG levels between normal, α-thal heterozygous, and α-thal homozygous children in the same age group (*P*-values not shown).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal and α-thal heterozygous children in the same age group (*P*-values not shown).

[£] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal and α-thal homozygous children in the same age group (*P*-values not shown).

[Δ] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between α-thal heterozygous and α-thal homozygous children in the same age group (*P*-values not shown).

Table 31 IgG responses to A4 schizont extract in the presence and absence of alpha-thalassemia in different age groups.

Schizont extract		Normal					Alpha-Thalassemia HETEROZYGOUS					Alpha-Thalassemia HOMOZYGOUS				
		0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 - 3 years	4 - 6 years	7 - 10 years	P^3	0 years	1 – 3 years	4 – 6 years	7 – 10 years	P^3
May 2002[1]	N[2]	10	43	32	5		14	56	64	16		5	24	21	1	
	Median	0.23[ε]	0.32	0.85	0.41	**0.014**	0.14	0.18	0.55	0.9	S*	0.07[ε]	0.15	0.6	0.37	**0.001**
	IQR	(0.11 - 0.41)	(0.1 - 0.8)	(0.32 - 1.28)	(0.23 - 1.23)		(0.06 - 0.43)	(0.07 - 0.54)	(0.27 - 1.04)	(0.59 - 1.17)		(0.07 - 0.08)	(0.09 - 0.43)	(0.34 - 0.88)	(0.37 - 0.37)	
October 2002[1]	N[2]	15	39	27	10		14	41	62	20		3	27	18	6	
	p50[Δ]	0.05	0.1	0.33	0.22	**0.002***	0.1	0.04	0.27	0.39	S*	0.19	0.07	0.22	0.14	**0.011***
	IQR	(0.01 - 0.21)	(0.03 - 0.18)	(0.08 - 0.65)	(0.12 - 1.03)		(0.07 - 0.17)	(0.01 - 0.15)	(0.11 - 0.59)	(0.18 - 0.91)		(-0.02 - 0.61)	(0.02 - 0.13)	(0.08 - 0.31)	(0.08 - 0.64)	
May 2003[1]	N[2]	7	36	33	14		10	39	57	30		6	24	15	7	
	Median	0.05	0.12	0.38	0.4	**0.046***	0.03	0.08	0.19	0.59	S*	0.1	0.08	0.16	0.35	0.082*
	IQR	(0.01 - 0.94)	(0.06 - 0.23)	(0.06 - 0.85)	(0.13 - 0.93)		(0.02 - 0.18)	(0.03 - 0.3)	(0.09 - 0.43)	(0.45 - 1)		(0.02 - 0.31)	(0.05 - 0.17)	(0.04 - 0.48)	(0.09 - 0.84)	
October 2003[1]	N[2]	6	35	35	17		8	40	52	32		4	20	19	7	
	Median	0.13	0.16	0.33	0.51	**0.026***	0.06	0.08	0.27	0.56	S*	0.09	0.07	0.15	0.49	**0.024***
	IQR	(0.08 - 0.18)	(0.03 - 0.73)	(0.13 - 1.04)	(0.36 - 1.1)		(0.02 - 0.3)	(0.03 - 0.3)	(0.11 - 0.94)	(0.28 - 0.95)		(0.04 - 0.13)	(0.04 - 0.27)	(0.1 - 0.48)	(0.16 - 0.78)	
May 2004[1]	N[2]	3	34	29	22		2	38	38	45		1	19	18	11	
	Median	0.12	0.16	0.05	0.03	**0.002***	0.06	0.18	0.09	0.04	**0.003***	0.06	0.2	0.06	0.04	0.058*
	IQR	(0.12 - 1.2)	(0.1 - 0.33)	(0.02 - 0.26)	(0.01 - 0.12)		(0.03 - 0.09)	(0.09 - 0.3)	(0.03 - 0.29)	(0.01 - 0.1)		(0.06 - 0.06)	(0.05 - 0.85)	(0.01 - 0.1)	(0.01 - 0.08)	
October 2004[1]	N[2]	-	26	31	26		-	30	39	47		-	17	15	14	
	Median	-	0.03	0.12	0.22[ε]	S*	-	0.02	0.07	0.19	S*	-	0.04	0.08	0.14[ε]	**0.048***
	IQR	-	(0.01 - 0.1)	(0.05 - 0.26)	(0.11 - 0.51)		-	(0.01 - 0.06)	(0.03 - 0.22)	(0.1 - 0.49)		-	(0.03 - 0.06)	(0.05 - 0.17)	(0.05 - 0.23)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type, *S* = *P* < 0.001).

* Significant difference (*P* ≤ 0.05 Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

† Significant difference (*P* ≤ 0.05 Kruskal Wallis) in median IgG levels between normal, α-thal heterozygous, and α-thal homozygous children in the same age group (*P*-values not shown).

‡ Significant difference (*P* ≤ 0.05 Wilcoxon rank test) in median IgG levels between normal and α-thal heterozygous children in the same age group (*P*-values not shown).

ε Significant difference (*P* ≤ 0.05 Wilcoxon rank test) in median IgG levels between normal and α-thal homozygous children in the same age group (*P*-values not shown).

Σ Significant difference (*P* ≤ 0.05 Wilcoxon rank test) in median IgG levels between α-thal heterozygous and α-thal homozygous children in the same age group (*P*-values not shown).

Table 32 IgG responses to tetanus toxoid in the presence and absence of alpha-thalassemia in different age groups.

Tetanus toxoid		Normal					Alpha-Thalassemia HETEROZYGOUS					Alpha-Thalassemia HOMOZYGOUS				
		0 years	1 - 3 years	4 - 6 years	7 - 10 years	P[3]	0 years	1 - 3 years	4 - 6 years	7 - 10 years	P[3]	0 years	1 – 3 years	4 – 6 years	7 – 10 years	P[3]
May 2002[1]	N[2]	10	44	32	5		14	55	64	16		5	25	21	1	
	Median	0.77	0.67	0.41	0.41	**0.08**	0.74	0.62	0.49	0.32	0.145*	1.68	0.87	0.39	0.62	**0.004**
	IQR	(0.16 - 1.37)	(0.43 - 1.08)	(0.29 - 0.71)	(0.35 - 0.47)		(0.44 - 1.2)	(0.32 - 0.99)	(0.26 - 0.78)	(0.18 - 0.57)		(1.43 - 1.83)	(0.39 - 1.11)	(0.16 - 0.58)	(0.62 - 0.62)	
October 2002[1]	N[2]	15	39	27	10		14	40	62	20		3	27	18	6	
	p50[Δ]	1.17	0.71	0.49	0.44	0.157	1.38	0.75	0.45	0.34	**0.035***	1.64	0.85	0.55	0.43	0.066
	IQR	(0.15 - 2.11)	(0.39 - 1.11)	(0.32 - 0.68)	(0.35 - 0.59)		(0.27 - 2.2)	(0.44 - 0.96)	(0.3 - 0.72)	(0.23 - 0.71)		(1.1 - 2.45)	(0.36 - 1.33)	(0.36 - 1.02)	(0.32 - 0.6)	
May 2003[1]	N[2]	7	35	32	13		10	39	57	30		6	24	15	7	
	Median	1.53	0.63	0.51	0.41	**0.017**	1.31	0.57	0.49	0.35	**0.023**	1.37	0.71	0.45	0.53	0.328
	IQR	(1.13 - 2.32)	(0.29 - 0.86)	(0.29 - 1.05)	(0.28 - 0.75)		(0.84 - 1.81)	(0.29 - 1.05)	(0.25 - 0.8)	(0.19 - 0.81)		(1.09 - 1.97)	(0.28 - 1.16)	(0.23 - 1.64)	(0.14 - 2.18)	
October 2003[1]	N[2]	7	35	36	17		9	40	54	33		4	22	19	7	
	Median	0.38	0.63	0.5	0.33	0.944	1.93	0.59	0.4	0.32	S*	0.75	0.65	0.7	0.44	0.945
	IQR	(0.13 - 2.12)	(0.21 - 1.09)	(0.23 - 0.86)	(0.22 - 1)		(1.36 - 2.27)	(0.38 - 0.93)	(0.25 - 0.75)	(0.22 - 0.53)		(0.51 - 1.44)	(0.5 - 1.03)	(0.36 - 1.09)	(0.27 - 1.57)	
May 2004[1]	N[2]	3	34	28	22		2	37	38	45		1	19	18	11	
	Median	0.91	0.6	0.69	0.35	0.466	2.43	0.62	0.45	0.35	**0.022**	0.68	0.56	0.38	1.05	0.397
	IQR	(0.29 - 2.18)	(0.23 - 0.92)	(0.36 - 1)	(0.23 - 0.71)		(2.35 - 2.51)	(0.4 - 0.92)	(0.26 - 0.67)	(0.23 - 0.51)		(0.68 - 0.68)	(0.43 - 1.03)	(0.26 - 0.96)	(0.26 - 2.22)	
October 2004[1]	N[2]	-	26	31	26		-	30	39	47		-	17	15	14	
	Median	-	0.68	0.7	0.42	0.685	-	0.53	0.58	0.37	0.647	-	0.72	0.39	0.83	0.217
	IQR	-	(0.31 - 1.19)	(0.28 - 1.15)	(0.32 - 0.68)		-	(0.34 - 1.15)	(0.29 - 0.85)	(0.27 - 1.16)		-	(0.54 - 1.04)	(0.28 - 0.8)	(0.31 - 1.83)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA, missing values are due to a lack of children in that category.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type, S = $P < 0.001$).

* Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children ages 1-3 years and children ages 7-10 years (*P*-values not shown).

† Significant difference ($P \leq 0.05$ Kruskal Wallis) in median IgG levels between normal, α-thal heterozygous, and α-thal homozygous children in the same age group (*P*-values not shown).

‡ Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal and α-thal heterozygous children in the same age group (*P*-values not shown).

[6] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal and α-thal homozygous children in the same age group (*P*-values not shown).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between α-thal heterozygous and α-thal homozygous children in the same age group (*P*-values not shown).

Table 33A Effect of different factors on IgG responses to AMA1(HB3) in the May 2002 cross sectional bleed.

	AMA1(HB3) May 2002		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.13 (0.09-0.16)	**<0.001**	298
Parasitemia	0.69 (0.48-0.89)	**<0.001**	298
Alpha-thal HET	-0.06 (-0.24-0.13)	0.533	297
Alpha-thal HOM	-0.26 (-0.5--0.01)	**0.038**	
Sickle trait	-0.23 (-0.46-0.01)	0.064	299
Prior parasitemia	0.32 (0.23-0.41)	**<0.001**	304
Prior episode	0.36 (0.2-0.51)	**<0.001**	304
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.09 (0.06-0.13)	**<0.001**	289
Parasitemia	0.42 (0.22-0.62)	**<0.001**	
Alpha-thal HET	-0.04 (-0.21-0.12)	0.606	
Alpha-thal HOM	-0.15 (-0.37-0.06)	0.155	
Sickle trait	-0.19 (-0.4-0.02)	0.074	
Prior parasitemia	0.22 (0.13-0.3)	**<0.001**	
Parasite negative[7]			
Age (years)	0.08 (0.04-0.11)	**<0.001**	239
Alpha-thal HET	-0.02 (-0.18-0.14)	0.799	
Alpha-thal HOM	-0.15 (-0.35-0.06)	0.159	
Sickle trait	-0.12 (-0.33-0.09)	0.269	
Prior parasitemia	0.3 (0.2-0.39)	**<0.001**	
Parasite positive[8]			
Age (years)	0.14 (-0.01-0.29)	0.073	50
Alpha-thal HET	-0.32 (-0.9-0.25)	0.263	
Alpha-thal HOM	-0.36 (-1.33-0.61)	0.457	
Sickle trait	-0.39 (-1.02-0.25)	0.226	
Prior parasitemia	0 (-0.27-0.26)	0.982	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.1 (0.07-0.14)	**<0.001**	289
Parasitemia	0.46 (0.25-0.66)	**<0.001**	
Alpha-thal HET	-0.07 (-0.24-0.1)	0.43	
Alpha-thal HOM	-0.16 (-0.38-0.06)	0.146	
Sickle trait	-0.19 (-0.4-0.03)	0.086	
Prior episode	0.23 (0.09-0.38)	**0.002**	
Parasite negative[7]			
Age (years)	0.1 (0.06-0.13)	**<0.001**	239
Alpha-thal HET	-0.03 (-0.21-0.14)	0.689	
Alpha-thal HOM	-0.15 (-0.37-0.06)	0.162	
Sickle trait	-0.15 (-0.37-0.07)	0.175	
Prior episode	0.29 (0.13-0.45)	**0.001**	
Parasite positive[8]			
Age (years)	0.14 (-0.01-0.3)	0.063	50
Alpha-thal HET	-0.3 (-0.86-0.27)	0.294	
Alpha-thal HOM	-0.31 (-1.31-0.68)	0.53	
Sickle trait	-0.36 (-1.01-0.29)	0.272	
Prior episode	0.08 (-0.33-0.49)	0.711	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 33B Effect of different factors on IgG responses to AMA1(HB3) in the October 2002 cross sectional bleed.

	AMA1(HB3) Oct 2002		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.07 (0.04-0.1)	**<0.001**	291
Parasitemia	0.39 (0.12-0.66)	**0.004**	291
Alpha-thal HET	-0.01 (-0.16-0.15)	0.93	284
Alpha-thal HOM	-0.23 (-0.43--0.03)	**0.026**	
Sickle trait	-0.2 (-0.41-0.02)	0.069	292
Prior parasitemia	0.23 (0.09-0.36)	**0.001**	254
Prior episode	0.26 (0.09-0.43)	**0.003**	254
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.11 (0.08-0.14)	**<0.001**	251
Parasitemia	0.26 (0.01-0.51)	**0.042**	
Alpha-thal HET	-0.03 (-0.18-0.12)	0.7	
Alpha-thal HOM	-0.17 (-0.37-0.02)	0.077	
Sickle trait	-0.15 (-0.36-0.05)	0.149	
Prior parasitemia	0.19 (0.07-0.31)	**0.002**	
Parasite negative[7]			
Age (years)	0.11 (0.08-0.14)	**<0.001**	232
Alpha-thal HET	0 (-0.15-0.16)	0.983	
Alpha-thal HOM	-0.12 (-0.32-0.07)	0.201	
Sickle trait	-0.13 (-0.34-0.08)	0.214	
Prior parasitemia	0.22 (0.09-0.34)	**0.001**	
Parasite positive[8]			
Age (years)	0.09 (-0.19-0.37)	0.486	19
Alpha-thal HET	-0.2 (-1.4-0.99)	0.721	
Alpha-thal HOM	-0.97 (-2.42-0.49)	0.175	
Sickle trait	-0.59 (-1.89-0.72)	0.348	
Prior parasitemia	-0.15 (-0.93-0.63)	0.684	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.11 (0.07-0.14)	**<0.001**	251
Parasitemia	0.28 (0.03-0.53)	**0.03**	
Alpha-thal HET	-0.04 (-0.19-0.11)	0.622	
Alpha-thal HOM	-0.18 (-0.37-0.01)	0.069	
Sickle trait	-0.17 (-0.38-0.04)	0.114	
Prior episode	0.21 (0.05-0.37)	**0.009**	
Parasite negative[7]			
Age (years)	0.11 (0.08-0.14)	**<0.001**	232
Alpha-thal HET	-0.01 (-0.17-0.14)	0.853	
Alpha-thal HOM	-0.13 (-0.33-0.06)	0.175	
Sickle trait	-0.15 (-0.35-0.06)	0.17	
Prior episode	0.24 (0.08-0.4)	**0.003**	
Parasite positive[8]			
Age (years)	0.1 (-0.16-0.37)	0.406	19
Alpha-thal HET	-0.28 (-1.38-0.83)	0.599	
Alpha-thal HOM	-0.97 (-2.43-0.49)	0.173	
Sickle trait	-0.55 (-1.83-0.73)	0.368	
Prior episode	-0.14 (-0.97-0.68)	0.714	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 33C Effect of different factors on IgG responses to AMA1(HB3) in the May 2003 cross sectional bleed.

	AMA1(HB3) May 2003		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.11 (0.07-0.14)	**<0.001**	285
Parasitemia	0.56 (0.32-0.79)	**<0.001**	285
Alpha-thal HET	0.04 (-0.15-0.23)	0.694	279
Alpha-thal HOM	-0.15 (-0.4-0.09)	0.22	
Sickle trait	-0.29 (-0.54--0.04)	0.021	283
Prior parasitemia	0.07 (0-0.14)	**0.047**	253
Prior episode	0.12 (0.02-0.21)	**0.015**	253
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.1 (0.06-0.14)	**<0.001**	247
Parasitemia	0.44 (0.19-0.69)	**0.001**	
Alpha-thal HET	0.03 (-0.17-0.22)	0.784	
Alpha-thal HOM	-0.06 (-0.31-0.18)	0.612	
Sickle trait	-0.27 (-0.53--0.01)	**0.043**	
Prior parasitemia	0.05 (-0.02-0.11)	0.158	
Parasite negative[7]			
Age (years)	0.08 (0.04-0.12)	**<0.001**	213
Alpha-thal HET	0.09 (-0.11-0.3)	0.367	
Alpha-thal HOM	-0.05 (-0.3-0.2)	0.697	
Sickle trait	-0.17 (-0.44-0.09)	0.205	
Prior parasitemia	0.07 (0.01-0.14)	**0.031**	
Parasite positive[8]			
Age (years)	0.12 (-0.01-0.25)	0.072	34
Alpha-thal HET	-0.22 (-0.83-0.4)	0.474	
Alpha-thal HOM	0.5 (-0.63-1.64)	0.373	
Sickle trait	-1.05 (-2.14-0.04)	0.059	
Prior parasitemia	-0.12 (-0.37-0.13)	0.323	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.09 (0.05-0.13)	**<0.001**	247
Parasitemia	0.46 (0.21-0.71)	**<0.001**	
Alpha-thal HET	0.03 (-0.17-0.22)	0.769	
Alpha-thal HOM	-0.07 (-0.31-0.18)	0.597	
Sickle trait	-0.26 (-0.52-0)	0.047	
Prior episode	0.09 (0-0.18)	0.052	
Parasite negative[7]			
Age (years)	0.08 (0.04-0.12)	**<0.001**	213
Alpha-thal HET	0.09 (-0.11-0.3)	0.369	
Alpha-thal HOM	-0.05 (-0.3-0.19)	0.671	
Sickle trait	-0.18 (-0.44-0.09)	0.19	
Prior episode	0.11 (0.02-0.2)	**0.015**	
Parasite positive[8]			
Age (years)	0.13 (0-0.25)	0.055	34
Alpha-thal HET	-0.25 (-0.85-0.36)	0.409	
Alpha-thal HOM	0.69 (-0.5-1.88)	0.246	
Sickle trait	-1.21 (-2.31--0.12)	0.031	
Prior episode	-0.3 (-0.75-0.16)	0.193	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 33D Effect of different factors on IgG responses to AMA1(HB3) in the October 2003 cross sectional bleed.

	AMA1(HB3) Oct 2003		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.12 (0.09-0.15)	**<0.001**	294
Parasitemia	1.09 (0.89-1.3)	**<0.001**	293
Alpha-thal HET	-0.09 (-0.28-0.1)	0.339	285
Alpha-thal HOM	-0.28 (-0.53--0.04)	**0.023**	
Sickle trait	-0.24 (-0.49-0.01)	0.059	284
Prior parasitemia	0.25 (0.15-0.34)	**<0.001**	261
Prior episode	0.25 (0.1-0.39)	**0.001**	261
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.11 (0.08-0.14)	**<0.001**	248
Parasitemia	0.89 (0.69-1.1)	**<0.001**	
Alpha-thal HET	-0.1 (-0.27-0.06)	0.2	
Alpha-thal HOM	-0.11 (-0.32-0.09)	0.281	
Sickle trait	-0.28 (-0.49--0.08)	**0.007**	
Prior parasitemia	0.18 (0.1-0.26)	**<0.001**	
Parasite negative[7]			
Age (years)	0.1 (0.07-0.13)	**<0.001**	212
Alpha-thal HET	-0.07 (-0.23-0.1)	0.412	
Alpha-thal HOM	-0.05 (-0.25-0.15)	0.618	
Sickle trait	-0.15 (-0.36-0.05)	0.145	
Prior parasitemia	0.21 (0.12-0.29)	**<0.001**	
Parasite positive[8]			
Age (years)	0.19 (0.04-0.34)	**0.015**	36
Alpha-thal HET	-0.24 (-0.8-0.32)	0.387	
Alpha-thal HOM	-0.3 (-1.43-0.83)	0.589	
Sickle trait	-0.99 (-1.71--0.27)	**0.009**	
Prior parasitemia	0.13 (-0.18-0.44)	0.398	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.11 (0.08-0.14)	**<0.001**	248
Parasitemia	0.95 (0.75-1.16)	**<0.001**	
Alpha-thal HET	-0.1 (-0.26-0.06)	0.23	
Alpha-thal HOM	-0.08 (-0.29-0.13)	0.44	
Sickle trait	-0.28 (-0.49--0.07)	**0.01**	
Prior episode	0.2 (0.09-0.32)	**0.001**	
Parasite negative[7]			
Age (years)	0.1 (0.07-0.14)	**<0.001**	212
Alpha-thal HET	-0.08 (-0.25-0.09)	0.341	
Alpha-thal HOM	-0.04 (-0.25-0.16)	0.677	
Sickle trait	-0.13 (-0.34-0.09)	0.236	
Prior episode	0.21 (0.1-0.33)	**<0.001**	
Parasite positive[8]			
Age (years)	0.18 (0.04-0.33)	**0.013**	36
Alpha-thal HET	-0.2 (-0.74-0.33)	0.444	
Alpha-thal HOM	-0.13 (-1.19-0.94)	0.811	
Sickle trait	-1.05 (-1.75--0.35)	**0.005**	
Prior episode	0.25 (-0.19-0.69)	0.255	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 33E Effect of different factors on IgG responses to AMA1(HB3) in the May 2004 cross sectional bleed.

	AMA1(HB3) May 2004		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.1 (0.07-0.12)	**<0.001**	279
Parasitemia	0.84 (0.55-1.14)	**<0.001**	280
Alpha-thal HET	-0.05 (-0.22-0.13)	0.6	261
Alpha-thal HOM	-0.31 (-0.54--0.09)	**0.006**	
Sickle trait	-0.22 (-0.45-0.01)	0.056	266
Prior parasitemia	0.23 (0.06-0.41)	**0.008**	254
Prior episode	-	-	
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.1 (0.06-0.13)	**<0.001**	239
Parasitemia	0.52 (0.22-0.83)	**0.001**	
Alpha-thal HET	-0.03 (-0.19-0.14)	0.728	
Alpha-thal HOM	-0.19 (-0.4-0.01)	0.064	
Sickle trait	-0.21 (-0.42-0)	0.051	
Prior parasitemia	0.16 (0-0.33)	0.051	
Parasite negative[7]			
Age (years)	0.08 (0.05-0.11)	**<0.001**	224
Alpha-thal HET	-0.03 (-0.19-0.14)	0.765	
Alpha-thal HOM	-0.19 (-0.39-0.02)	0.078	
Sickle trait	-0.2 (-0.41-0.02)	0.069	
Prior parasitemia	0.26 (0.07-0.45)	**0.006**	
Parasite positive[8]			
Age (years)	0.27 (0.04-0.49)	**0.026**	15
Alpha-thal HET	-0.57 (-1.54-0.4)	0.218	
Alpha-thal HOM	-1.33 (-2.96-0.29)	0.095	
Sickle trait	-0.56 (-1.96-0.84)	0.391	
Prior parasitemia	0.02 (-0.62-0.66)	0.943	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.1 (0.07-0.13)	**<0.001**	239
Parasitemia	0.57 (0.27-0.88)	**<0.001**	
Alpha-thal HET	-0.04 (-0.21-0.12)	0.594	
Alpha-thal HOM	-0.2 (-0.41-0)	0.054	
Sickle trait	-0.21 (-0.42-0)	0.053	
Prior episode	-	-	
Parasite negative[7]			
Age (years)	0.09 (0.06-0.12)	**<0.001**	224
Alpha-thal HET	-0.04 (-0.21-0.13)	0.652	
Alpha-thal HOM	-0.19 (-0.4-0.02)	0.079	
Sickle trait	-0.19 (-0.41-0.03)	0.088	
Prior episode	-	-	
Parasite positive[8]			
Age (years)	0.26 (0.1-0.42)	**0.005**	15
Alpha-thal HET	-0.58 (-1.44-0.28)	0.163	
Alpha-thal HOM	-1.35 (-2.8-0.1)	0.064	
Sickle trait	-0.58 (-1.66-0.49)	0.255	
Prior episode	-	-	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 33F Effect of different factors on IgG responses to AMA1(HB3) in the October 2004 cross sectional bleed.

	AMA1(HB3) Oct 2004		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.07 (0.05-0.09)	**<0.001**	273
Parasitemia	0.69 (0.37-1.01)	**<0.001**	273
Alpha-thal HET	-0.05 (-0.2-0.1)	0.543	268
Alpha-thal HOM	-0.17 (-0.36-0.02)	0.079	
Sickle trait	-0.1 (-0.29-0.09)	0.3	270
Prior parasitemia	0.26 (-0.1-0.62)	0.161	235
Prior episode	-	-	
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.09 (0.06-0.12)	**<0.001**	216
Parasitemia	0.72 (0.34-1.1)	**<0.001**	
Alpha-thal HET	-0.06 (-0.21-0.09)	0.436	
Alpha-thal HOM	-0.17 (-0.36-0.02)	0.076	
Sickle trait	-0.13 (-0.32-0.06)	0.17	
Prior parasitemia	0.27 (-0.06-0.61)	0.104	
Parasite negative[7]			
Age (years)	0.09 (0.06-0.12)	**<0.001**	209
Alpha-thal HET	-0.07 (-0.22-0.09)	0.39	
Alpha-thal HOM	-0.18 (-0.37-0.01)	0.068	
Sickle trait	-0.1 (-0.29-0.1)	0.334	
Prior parasitemia	0.28 (-0.05-0.61)	0.099	
Parasite positive[8]			
Age (years)	0.1 (-0.58-0.78)	0.587	7
Alpha-thal HET	-0.3 (-4.52-3.91)	0.785	
Alpha-thal HOM	0.39 (-5.03-5.81)	0.785	
Sickle trait	-0.93 (-4.93-3.06)	0.421	
Prior parasitemia	-	-	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.09 (0.06-0.12)	**<0.001**	216
Parasitemia	0.72 (0.34-1.1)	**<0.001**	
Alpha-thal HET	-0.05 (-0.2-0.1)	0.523	
Alpha-thal HOM	-0.15 (-0.34-0.04)	0.114	
Sickle trait	-0.13 (-0.32-0.06)	0.169	
Prior episode	-	-	
Parasite negative[7]			
Age (years)	0.09 (0.06-0.12)	**<0.001**	209
Alpha-thal HET	-0.06 (-0.21-0.1)	0.475	
Alpha-thal HOM	-0.16 (-0.35-0.03)	0.104	
Sickle trait	-0.1 (-0.29-0.1)	0.332	
Prior episode	-	-	
Parasite positive[8]			
Age (years)	0.1 (-0.58-0.78)	0.587	7
Alpha-thal HET	-0.3 (-4.52-3.91)	0.785	
Alpha-thal HOM	0.39 (-5.03-5.81)	0.785	
Sickle trait	-0.93 (-4.93-3.06)	0.421	
Prior episode	-	-	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 34A Effect of different factors on IgG responses to AMA1(3D7) in the May 2002 cross sectional bleed.

	AMA1(3D7) May 2002		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.11 (0.08-0.15)	**<0.001**	298
Parasitemia	0.61 (0.42-0.8)	**<0.001**	298
Alpha-thal HET	-0.04 (-0.21-0.13)	0.636	297
Alpha-thal HOM	-0.24 (-0.46--0.02)	**0.036**	
Sickle trait	-0.21 (-0.43-0.01)	0.055	299
Prior parasitemia	0.29 (0.21-0.37)	**<0.001**	304
Prior episode	0.33 (0.19-0.47)	**<0.001**	304
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.08 (0.05-0.11)	**<0.001**	289
Parasitemia	0.37 (0.19-0.55)	**<0.001**	
Alpha-thal HET	-0.03 (-0.18-0.13)	0.733	
Alpha-thal HOM	-0.14 (-0.34-0.05)	0.148	
Sickle trait	-0.18 (-0.37-0.01)	0.065	
Prior parasitemia	0.2 (0.11-0.28)	**<0.001**	
Parasite negative[7]			
Age (years)	0.07 (0.04-0.1)	**<0.001**	239
Alpha-thal HET	0.01 (-0.14-0.15)	0.937	
Alpha-thal HOM	-0.11 (-0.29-0.07)	0.242	
Sickle trait	-0.12 (-0.31-0.07)	0.199	
Prior parasitemia	0.27 (0.18-0.35)	**<0.001**	
Parasite positive[8]			
Age (years)	0.12 (-0.03-0.26)	0.124	50
Alpha-thal HET	-0.33 (-0.89-0.24)	0.249	
Alpha-thal HOM	-0.57 (-1.52-0.38)	0.236	
Sickle trait	-0.32 (-0.95-0.3)	0.302	
Prior parasitemia	-0.01 (-0.28-0.25)	0.914	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.09 (0.06-0.13)	**<0.001**	289
Parasitemia	0.41 (0.22-0.59)	**<0.001**	
Alpha-thal HET	-0.05 (-0.2-0.11)	0.547	
Alpha-thal HOM	-0.15 (-0.35-0.05)	0.141	
Sickle trait	-0.18 (-0.37-0.02)	0.078	
Prior episode	0.22 (0.08-0.35)	**0.002**	
Parasite negative[7]			
Age (years)	0.09 (0.06-0.12)	**<0.001**	239
Alpha-thal HET	-0.01 (-0.16-0.15)	0.937	
Alpha-thal HOM	-0.11 (-0.31-0.08)	0.238	
Sickle trait	-0.15 (-0.35-0.04)	0.129	
Prior episode	0.27 (0.12-0.41)	**<0.001**	
Parasite positive[8]			
Age (years)	0.12 (-0.03-0.27)	0.111	50
Alpha-thal HET	-0.3 (-0.86-0.25)	0.275	
Alpha-thal HOM	-0.54 (-1.52-0.44)	0.277	
Sickle trait	-0.31 (-0.95-0.33)	0.337	
Prior episode	0.04 (-0.36-0.45)	0.829	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 34B Effect of different factors on IgG responses to AMA1(3D7) in the October 2002 cross sectional bleed.

	AMA1(3D7) Oct 2002		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.07 (0.05-0.1)	**<0.001**	291
Parasitemia	0.34 (0.08-0.59)	**0.01**	291
Alpha-thal HET	0.01 (-0.14-0.16)	0.862	284
Alpha-thal HOM	-0.2 (-0.39--0.01)	**0.039**	
Sickle trait	-0.21 (-0.41--0.01)	**0.039**	292
Prior parasitemia	0.2 (0.08-0.33)	**0.002**	254
Prior episode	0.24 (0.08-0.4)	**0.004**	254
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.1 (0.07-0.13)	**<0.001**	251
Parasitemia	0.19 (-0.05-0.43)	0.118	
Alpha-thal HET	-0.01 (-0.16-0.13)	0.848	
Alpha-thal HOM	-0.15 (-0.33-0.03)	0.109	
Sickle trait	-0.17 (-0.37-0.02)	0.084	
Prior parasitemia	0.17 (0.06-0.29)	0.004	
Parasite negative[7]			
Age (years)	0.1 (0.07-0.13)	**<0.001**	232
Alpha-thal HET	0.02 (-0.12-0.17)	0.768	
Alpha-thal HOM	-0.1 (-0.28-0.08)	0.279	
Sickle trait	-0.16 (-0.35-0.04)	0.124	
Prior parasitemia	0.19 (0.08-0.31)	**0.001**	
Parasite positive[8]			
Age (years)	0.17 (-0.08-0.42)	0.16	19
Alpha-thal HET	-0.48 (-1.54-0.57)	0.34	
Alpha-thal HOM	-1.07 (-2.36-0.21)	0.095	
Sickle trait	-0.57 (-1.71-0.58)	0.307	
Prior parasitemia	-0.04 (-0.72-0.65)	0.91	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.1 (0.07-0.13)	**<0.001**	251
Parasitemia	0.21 (-0.03-0.45)	0.089	
Alpha-thal HET	-0.02 (-0.17-0.12)	0.77	
Alpha-thal HOM	-0.15 (-0.34-0.03)	0.098	
Sickle trait	-0.19 (-0.38-0.01)	0.063	
Prior episode	0.19 (0.04-0.34)	**0.014**	
Parasite negative[7]			
Age (years)	0.1 (0.07-0.13)	**<0.001**	232
Alpha-thal HET	0.01 (-0.14-0.15)	0.922	
Alpha-thal HOM	-0.11 (-0.29-0.08)	0.246	
Sickle trait	-0.17 (-0.37-0.03)	0.096	
Prior episode	0.21 (0.06-0.36)	**0.007**	
Parasite positive[8]			
Age (years)	0.17 (-0.06-0.41)	0.129	19
Alpha-thal HET	-0.5 (-1.47-0.47)	0.284	
Alpha-thal HOM	-1.07 (-2.36-0.21)	0.095	
Sickle trait	-0.56 (-1.68-0.57)	0.306	
Prior episode	-0.03 (-0.76-0.7)	0.933	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 34C Effect of different factors on IgG responses to AMA1(3D7) in the May 2003 cross sectional bleed.

	AMA1(3D7) May 2003		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.1 (0.07-0.13)	**<0.001**	285
Parasitemia	0.55 (0.33-0.78)	**<0.001**	285
Alpha-thal HET	0.04 (-0.14-0.22)	0.682	279
Alpha-thal HOM	-0.14 (-0.38-0.09)	0.23	
Sickle trait	-0.27 (-0.51--0.03)	**0.029**	283
Prior parasitemia	0.06 (0-0.13)	0.069	253
Prior episode	0.11 (0.02-0.2)	**0.016**	253
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.1 (0.06-0.13)	**<0.001**	247
Parasitemia	0.44 (0.2-0.67)	**<0.001**	
Alpha-thal HET	0.03 (-0.16-0.21)	0.789	
Alpha-thal HOM	-0.05 (-0.29-0.18)	0.666	
Sickle trait	-0.27 (-0.52--0.02)	**0.032**	
Prior parasitemia	0.04 (-0.02-0.1)	0.222	
Parasite negative[7]			
Age (years)	0.08 (0.04-0.12)	**<0.001**	213
Alpha-thal HET	0.08 (-0.11-0.28)	0.398	
Alpha-thal HOM	-0.05 (-0.28-0.19)	0.702	
Sickle trait	-0.18 (-0.43-0.07)	0.158	
Prior parasitemia	0.07 (0-0.13)	**0.04**	
Parasite positive[8]			
Age (years)	0.15 (0.02-0.28)	**0.021**	34
Alpha-thal HET	-0.15 (-0.74-0.45)	0.616	
Alpha-thal HOM	0.53 (-0.57-1.63)	0.33	
Sickle trait	-0.91 (-1.96-0.15)	0.089	
Prior parasitemia	-0.14 (-0.38-0.1)	0.236	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.09 (0.06-0.13)	**<0.001**	247
Parasitemia	0.46 (0.22-0.7)	**<0.001**	
Alpha-thal HET	0.03 (-0.16-0.21)	0.774	
Alpha-thal HOM	-0.05 (-0.29-0.18)	0.654	
Sickle trait	-0.26 (-0.51--0.02)	0.038	
Prior episode	0.08 (0-0.17)	0.056	
Parasite negative[7]			
Age (years)	0.08 (0.04-0.12)	**<0.001**	213
Alpha-thal HET	0.08 (-0.11-0.27)	0.399	
Alpha-thal HOM	-0.05 (-0.28-0.18)	0.68	
Sickle trait	-0.18 (-0.43-0.07)	0.149	
Prior episode	0.1 (0.02-0.19)	**0.015**	
Parasite positive[8]			
Age (years)	0.16 (0.03-0.28)	**0.016**	34
Alpha-thal HET	-0.18 (-0.77-0.41)	0.53	
Alpha-thal HOM	0.68 (-0.48-1.84)	0.24	
Sickle trait	-1.07 (-2.14-0)	0.051	
Prior episode	-0.28 (-0.72-0.17)	0.212	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 34D Effect of different factors on IgG responses to AMA1(3D7) in the October 2003 cross sectional bleed.

	AMA1(3D7) Oct 2003		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.11 (0.08-0.14)	**<0.001**	294
Parasitemia	1.04 (0.84-1.24)	**<0.001**	293
Alpha-thal HET	-0.08 (-0.26-0.1)	0.377	285
Alpha-thal HOM	-0.27 (-0.5--0.04)	**0.023**	
Sickle trait	-0.21 (-0.45-0.03)	0.092	284
Prior parasitemia	0.23 (0.13-0.32)	**<0.001**	261
Prior episode	0.22 (0.08-0.36)	**0.002**	261
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.11 (0.08-0.14)	**<0.001**	248
Parasitemia	0.86 (0.67-1.06)	**<0.001**	
Alpha-thal HET	-0.1 (-0.25-0.06)	0.209	
Alpha-thal HOM	-0.1 (-0.29-0.1)	0.33	
Sickle trait	-0.25 (-0.44--0.05)	**0.015**	
Prior parasitemia	0.17 (0.09-0.24)	**<0.001**	
Parasite negative[7]			
Age (years)	0.09 (0.07-0.12)	**<0.001**	212
Alpha-thal HET	-0.07 (-0.23-0.08)	0.339	
Alpha-thal HOM	-0.04 (-0.23-0.15)	0.673	
Sickle trait	-0.12 (-0.31-0.08)	0.247	
Prior parasitemia	0.18 (0.1-0.26)	**<0.001**	
Parasite positive[8]			
Age (years)	0.24 (0.1-0.39)	**0.002**	36
Alpha-thal HET	-0.22 (-0.77-0.33)	0.424	
Alpha-thal HOM	-0.32 (-1.42-0.78)	0.56	
Sickle trait	-0.95 (-1.66--0.25)	**0.01**	
Prior parasitemia	0.22 (-0.08-0.52)	0.147	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.11 (0.08-0.14)	**<0.001**	248
Parasitemia	0.92 (0.72-1.12)	**<0.001**	
Alpha-thal HET	-0.09 (-0.25-0.06)	0.235	
Alpha-thal HOM	-0.07 (-0.27-0.13)	0.488	
Sickle trait	-0.24 (-0.44--0.04)	**0.019**	
Prior episode	0.18 (0.07-0.3)	**0.002**	
Parasite negative[7]			
Age (years)	0.1 (0.07-0.12)	**<0.001**	212
Alpha-thal HET	-0.09 (-0.24-0.07)	0.28	
Alpha-thal HOM	-0.03 (-0.23-0.16)	0.723	
Sickle trait	-0.09 (-0.3-0.11)	0.359	
Prior episode	0.18 (0.07-0.3)	**0.001**	
Parasite positive[8]			
Age (years)	0.23 (0.08-0.37)	**0.003**	36
Alpha-thal HET	-0.14 (-0.68-0.39)	0.584	
Alpha-thal HOM	-0.04 (-1.1-1.02)	0.943	
Sickle trait	-1.06 (-1.75--0.36)	**0.004**	
Prior episode	0.3 (-0.13-0.74)	0.162	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 34E Effect of different factors on IgG responses to AMA1(3D7) in the May 2004 cross sectional bleed.

	AMA1(3D7) May 2004		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.09 (0.06-0.11)	**<0.001**	279
Parasitemia	0.64 (0.36-0.92)	**<0.001**	280
Alpha-thal HET	0 (-0.16-0.16)	0.984	261
Alpha-thal HOM	-0.24 (-0.45--0.04)	**0.02**	
Sickle trait	-0.22 (-0.43-0)	**0.045**	266
Prior parasitemia	0.23 (0.07-0.39)	**0.004**	254
Prior episode	-	-	
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.09 (0.06-0.11)	**<0.001**	239
Parasitemia	0.33 (0.04-0.62)	**0.025**	
Alpha-thal HET	0.01 (-0.14-0.16)	0.892	
Alpha-thal HOM	-0.14 (-0.34-0.05)	0.143	
Sickle trait	-0.21 (-0.41--0.01)	**0.04**	
Prior parasitemia	0.19 (0.04-0.34)	**0.015**	
Parasite negative[7]			
Age (years)	0.08 (0.05-0.11)	**<0.001**	224
Alpha-thal HET	0.02 (-0.14-0.17)	0.84	
Alpha-thal HOM	-0.13 (-0.32-0.07)	0.192	
Sickle trait	-0.2 (-0.4-0)	0.054	
Prior parasitemia	0.24 (0.06-0.41)	**0.008**	
Parasite positive[8]			
Age (years)	0.25 (0.01-0.49)	**0.04**	15
Alpha-thal HET	-0.4 (-1.42-0.62)	0.398	
Alpha-thal HOM	-1.2 (-2.9-0.51)	0.147	
Sickle trait	-0.34 (-1.81-1.13)	0.616	
Prior parasitemia	0.23 (-0.45-0.9)	0.465	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.09 (0.06-0.12)	**<0.001**	239
Parasitemia	0.39 (0.1-0.67)	**0.008**	
Alpha-thal HET	-0.01 (-0.16-0.15)	0.924	
Alpha-thal HOM	-0.16 (-0.35-0.04)	0.119	
Sickle trait	-0.21 (-0.41--0.01)	0.044	
Prior episode	-	-	
Parasite negative[7]			
Age (years)	0.08 (0.05-0.11)	**<0.001**	224
Alpha-thal HET	0 (-0.15-0.16)	0.962	
Alpha-thal HOM	-0.13 (-0.33-0.07)	0.192	
Sickle trait	-0.19 (-0.39-0.01)	0.068	
Prior episode	-	-	
Parasite positive[8]			
Age (years)	0.2 (0.02-0.38)	**0.03**	15
Alpha-thal HET	-0.52 (-1.45-0.42)	0.246	
Alpha-thal HOM	-1.37 (-2.94-0.21)	0.082	
Sickle trait	-0.62 (-1.79-0.55)	0.265	
Prior episode	-	-	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 34F Effect of different factors on IgG responses to AMA1(3D7) in the October 2004 cross sectional bleed.

	AMA1(3D7) Oct 2004		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.06 (0.04-0.08)	**<0.001**	273
Parasitemia	0.48 (0.19-0.77)	**0.001**	273
Alpha-thal HET	-0.01 (-0.14-0.13)	0.925	268
Alpha-thal HOM	-0.13 (-0.3-0.04)	0.133	
Sickle trait	-0.12 (-0.29-0.05)	0.168	270
Prior parasitemia	0.22 (-0.1-0.54)	0.18	235
Prior episode	-	-	
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.08 (0.05-0.1)	**<0.001**	216
Parasitemia	0.49 (0.15-0.84)	**0.005**	
Alpha-thal HET	-0.01 (-0.15-0.12)	0.844	
Alpha-thal HOM	-0.13 (-0.3-0.04)	0.141	
Sickle trait	-0.15 (-0.32-0.02)	0.088	
Prior parasitemia	0.22 (-0.08-0.52)	0.144	
Parasite negative[7]			
Age (years)	0.08 (0.05-0.1)	**<0.001**	209
Alpha-thal HET	-0.03 (-0.17-0.1)	0.637	
Alpha-thal HOM	-0.14 (-0.31-0.03)	0.104	
Sickle trait	-0.12 (-0.29-0.06)	0.18	
Prior parasitemia	0.23 (-0.07-0.52)	0.131	
Parasite positive[8]			
Age (years)	0.04 (-0.66-0.73)	0.848	7
Alpha-thal HET	0.43 (-3.91-4.78)	0.709	
Alpha-thal HOM	0.17 (-5.42-5.76)	0.909	
Sickle trait	-0.59 (-4.71-3.53)	0.601	
Prior parasitemia	-	-	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.08 (0.05-0.1)	**<0.001**	216
Parasitemia	0.49 (0.14-0.83)	**0.006**	
Alpha-thal HET	0 (-0.14-0.13)	0.943	
Alpha-thal HOM	-0.11 (-0.28-0.06)	0.194	
Sickle trait	-0.15 (-0.32-0.02)	0.088	
Prior episode	-	-	
Parasite negative[7]			
Age (years)	0.08 (0.05-0.1)	**<0.001**	209
Alpha-thal HET	-0.02 (-0.16-0.11)	0.735	
Alpha-thal HOM	-0.13 (-0.3-0.05)	0.149	
Sickle trait	-0.12 (-0.29-0.06)	0.18	
Prior episode	-	-	
Parasite positive[8]			
Age (years)	0.04 (-0.66-0.73)	0.848	7
Alpha-thal HET	0.43 (-3.91-4.78)	0.709	
Alpha-thal HOM	0.17 (-5.42-5.76)	0.909	
Sickle trait	-0.59 (-4.71-3.53)	0.601	
Prior episode	-	-	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 35A Effect of different factors on IgG responses to MSP2(3D7) in the May 2002 cross sectional bleed.

	MSP2(3D7) May 2002		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.12 (0.08-0.15)	**<0.001**	298
Parasitemia	0.78 (0.59-0.98)	**<0.001**	298
Alpha-thal HET	0.01 (-0.17-0.19)	0.941	297
Alpha-thal HOM	-0.25 (-0.48--0.01)	**0.044**	
Sickle trait	-0.1 (-0.34-0.13)	0.393	299
Prior parasitemia	0.28 (0.19-0.36)	**<0.001**	304
Prior episode	0.33 (0.18-0.48)	**<0.001**	304
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.08 (0.04-0.11)	**<0.001**	289
Parasitemia	0.57 (0.37-0.76)	**<0.001**	
Alpha-thal HET	0.03 (-0.13-0.19)	0.704	
Alpha-thal HOM	-0.13 (-0.34-0.08)	0.217	
Sickle trait	-0.09 (-0.29-0.12)	0.396	
Prior parasitemia	0.18 (0.09-0.26)	**<0.001**	
Parasite negative[7]			
Age (years)	0.06 (0.03-0.09)	**<0.001**	239
Alpha-thal HET	0.01 (-0.14-0.16)	0.904	
Alpha-thal HOM	-0.12 (-0.31-0.07)	0.213	
Sickle trait	-0.07 (-0.26-0.13)	0.507	
Prior parasitemia	0.26 (0.17-0.35)	**<0.001**	
Parasite positive[8]			
Age (years)	0.12 (-0.04-0.29)	0.141	50
Alpha-thal HET	-0.05 (-0.68-0.57)	0.863	
Alpha-thal HOM	-0.55 (-1.6-0.5)	0.294	
Sickle trait	-0.1 (-0.79-0.59)	0.767	
Prior parasitemia	-0.03 (-0.32-0.26)	0.855	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.09 (0.05-0.12)	**<0.001**	289
Parasitemia	0.59 (0.4-0.79)	**<0.001**	
Alpha-thal HET	0.01 (-0.15-0.18)	0.864	
Alpha-thal HOM	-0.14 (-0.35-0.08)	0.208	
Sickle trait	-0.08 (-0.29-0.13)	0.439	
Prior episode	0.21 (0.07-0.35)	**0.004**	
Parasite negative[7]			
Age (years)	0.08 (0.05-0.11)	**<0.001**	239
Alpha-thal HET	0 (-0.17-0.16)	0.96	
Alpha-thal HOM	-0.13 (-0.33-0.08)	0.219	
Sickle trait	-0.1 (-0.31-0.11)	0.338	
Prior episode	0.21 (0.06-0.36)	**0.005**	
Parasite positive[8]			
Age (years)	0.14 (-0.02-0.31)	0.082	50
Alpha-thal HET	0.05 (-0.56-0.65)	0.879	
Alpha-thal HOM	-0.39 (-1.46-0.67)	0.462	
Sickle trait	-0.01 (-0.71-0.68)	0.97	
Prior episode	0.25 (-0.19-0.69)	0.261	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 35B Effect of different factors on IgG responses to MSP2(3D7) in the October 2002 cross sectional bleed.

	MSP2(3D7) Oct 2002		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.05 (0.03-0.07)	**<0.001**	291
Parasitemia	0.52 (0.35-0.7)	**<0.001**	291
Alpha-thal HET	-0.02 (-0.13-0.09)	0.691	284
Alpha-thal HOM	-0.14 (-0.28-0)	**0.045**	
Sickle trait	-0.05 (-0.19-0.1)	0.512	292
Prior parasitemia	0.05 (-0.04-0.15)	0.28	254
Prior episode	0.02 (-0.1-0.14)	0.748	254
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.05 (0.03-0.08)	**<0.001**	251
Parasitemia	0.49 (0.3-0.67)	**<0.001**	
Alpha-thal HET	-0.04 (-0.15-0.07)	0.528	
Alpha-thal HOM	-0.11 (-0.25-0.03)	0.134	
Sickle trait	-0.07 (-0.22-0.08)	0.361	
Prior parasitemia	0.02 (-0.07-0.11)	0.639	
Parasite negative[7]			
Age (years)	0.05 (0.03-0.07)	**<0.001**	232
Alpha-thal HET	-0.03 (-0.13-0.07)	0.542	
Alpha-thal HOM	-0.08 (-0.2-0.05)	0.222	
Sickle trait	-0.11 (-0.24-0.02)	0.094	
Prior parasitemia	0.02 (-0.06-0.1)	0.587	
Parasite positive[8]			
Age (years)	0.26 (-0.04-0.56)	0.084	19
Alpha-thal HET	-0.65 (-1.94-0.63)	0.292	
Alpha-thal HOM	-1.12 (-2.68-0.45)	0.149	
Sickle trait	0.36 (-1.04-1.77)	0.586	
Prior parasitemia	0.24 (-0.6-1.08)	0.553	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.05 (0.03-0.08)	**<0.001**	251
Parasitemia	0.49 (0.31-0.67)	**<0.001**	
Alpha-thal HET	-0.04 (-0.15-0.07)	0.49	
Alpha-thal HOM	-0.11 (-0.25-0.03)	0.118	
Sickle trait	-0.07 (-0.22-0.07)	0.326	
Prior episode	-0.01 (-0.13-0.1)	0.797	
Parasite negative[7]			
Age (years)	0.05 (0.03-0.07)	**<0.001**	232
Alpha-thal HET	-0.04 (-0.13-0.06)	0.456	
Alpha-thal HOM	-0.08 (-0.21-0.04)	0.175	
Sickle trait	-0.12 (-0.25-0.01)	0.071	
Prior episode	-0.06 (-0.16-0.04)	0.233	
Parasite positive[8]			
Age (years)	0.27 (0.01-0.54)	**0.043**	19
Alpha-thal HET	-0.59 (-1.69-0.51)	0.267	
Alpha-thal HOM	-1.09 (-2.55-0.37)	0.131	
Sickle trait	0.36 (-0.92-1.64)	0.553	
Prior episode	0.6 (-0.22-1.43)	0.138	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 35C Effect of different factors on IgG responses to MSP2(3D7) in the May 2003 cross sectional bleed.

	MSP2(3D7) May 2003		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.06 (0.04-0.09)	**<0.001**	285
Parasitemia	0.65 (0.49-0.81)	**<0.001**	285
Alpha-thal HET	-0.11 (-0.25-0.02)	0.105	279
Alpha-thal HOM	-0.18 (-0.36-0)	**0.049**	
Sickle trait	-0.07 (-0.26-0.12)	0.462	283
Prior parasitemia	0.03 (-0.02-0.08)	0.266	253
Prior episode	0.05 (-0.02-0.12)	0.147	253
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.05 (0.03-0.08)	**<0.001**	247
Parasitemia	0.53 (0.36-0.7)	**<0.001**	
Alpha-thal HET	-0.08 (-0.21-0.06)	0.259	
Alpha-thal HOM	-0.08 (-0.24-0.09)	0.376	
Sickle trait	-0.1 (-0.27-0.08)	0.284	
Prior parasitemia	0.01 (-0.03-0.05)	0.653	
Parasite negative[7]			
Age (years)	0.04 (0.02-0.06)	**0.001**	213
Alpha-thal HET	-0.05 (-0.16-0.07)	0.437	
Alpha-thal HOM	-0.06 (-0.2-0.08)	0.398	
Sickle trait	0 (-0.15-0.15)	0.996	
Prior parasitemia	0.05 (0.01-0.08)	**0.016**	
Parasite positive[8]			
Age (years)	0.11 (-0.01-0.23)	0.077	34
Alpha-thal HET	-0.08 (-0.67-0.5)	0.779	
Alpha-thal HOM	0.31 (-0.77-1.39)	0.558	
Sickle trait	-0.6 (-1.64-0.43)	0.241	
Prior parasitemia	-0.23 (-0.47-0)	0.052	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.05 (0.03-0.08)	**<0.001**	247
Parasitemia	0.54 (0.37-0.71)	**<0.001**	
Alpha-thal HET	-0.08 (-0.21-0.06)	0.261	
Alpha-thal HOM	-0.08 (-0.24-0.09)	0.375	
Sickle trait	-0.09 (-0.27-0.09)	0.326	
Prior episode	0.04 (-0.02-0.1)	0.215	
Parasite negative[7]			
Age (years)	0.04 (0.01-0.06)	**0.002**	213
Alpha-thal HET	-0.05 (-0.16-0.07)	0.431	
Alpha-thal HOM	-0.06 (-0.2-0.08)	0.375	
Sickle trait	0 (-0.15-0.15)	0.976	
Prior episode	0.07 (0.02-0.12)	**0.006**	
Parasite positive[8]			
Age (years)	0.12 (0-0.25)	0.054	34
Alpha-thal HET	-0.14 (-0.72-0.44)	0.626	
Alpha-thal HOM	0.53 (-0.61-1.68)	0.349	
Sickle trait	-0.86 (-1.91-0.2)	0.107	
Prior episode	-0.43 (-0.87-0.01)	0.054	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 35D Effect of different factors on IgG responses to MSP2(3D7) in the October 2003 cross sectional bleed.

	MSP2(3D7) Oct 2003		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.06 (0.04-0.09)	**<0.001**	294
Parasitemia	0.63 (0.48-0.79)	**<0.001**	293
Alpha-thal HET	-0.03 (-0.17-0.1)	0.621	285
Alpha-thal HOM	-0.13 (-0.3-0.05)	0.148	
Sickle trait	-0.1 (-0.28-0.08)	0.279	284
Prior parasitemia	0.12 (0.05-0.19)	**0.001**	261
Prior episode	0.05 (-0.05-0.16)	0.323	261
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.05 (0.03-0.08)	**<0.001**	248
Parasitemia	0.5 (0.34-0.67)	**<0.001**	
Alpha-thal HET	-0.04 (-0.17-0.09)	0.567	
Alpha-thal HOM	-0.04 (-0.21-0.12)	0.616	
Sickle trait	-0.1 (-0.26-0.07)	0.256	
Prior parasitemia	0.08 (0.02-0.15)	**0.013**	
Parasite negative[7]			
Age (years)	0.04 (0.02-0.06)	**<0.001**	212
Alpha-thal HET	0 (-0.12-0.12)	0.993	
Alpha-thal HOM	0 (-0.15-0.15)	0.991	
Sickle trait	0.03 (-0.13-0.18)	0.726	
Prior parasitemia	0.13 (0.07-0.19)	**<0.001**	
Parasite positive[8]			
Age (years)	0.13 (-0.01-0.27)	0.066	36
Alpha-thal HET	-0.09 (-0.61-0.43)	0.726	
Alpha-thal HOM	0.31 (-0.74-1.35)	0.554	
Sickle trait	-0.9 (-1.57--0.24)	**0.01**	
Prior parasitemia	-0.12 (-0.41-0.16)	0.377	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.05 (0.03-0.08)	**<0.001**	248
Parasitemia	0.53 (0.37-0.7)	**<0.001**	
Alpha-thal HET	-0.04 (-0.17-0.09)	0.548	
Alpha-thal HOM	-0.04 (-0.21-0.13)	0.649	
Sickle trait	-0.1 (-0.27-0.07)	0.235	
Prior episode	0.04 (-0.06-0.13)	0.453	
Parasite negative[7]			
Age (years)	0.04 (0.02-0.07)	**<0.001**	212
Alpha-thal HET	-0.01 (-0.14-0.11)	0.824	
Alpha-thal HOM	-0.01 (-0.16-0.15)	0.91	
Sickle trait	0.03 (-0.13-0.2)	0.686	
Prior episode	0.08 (-0.01-0.17)	0.099	
Parasite positive[8]			
Age (years)	0.14 (0.01-0.28)	**0.037**	36
Alpha-thal HET	-0.14 (-0.64-0.37)	0.583	
Alpha-thal HOM	0.15 (-0.85-1.16)	0.756	
Sickle trait	-0.84 (-1.5--0.18)	**0.014**	
Prior episode	-0.1 (-0.52-0.31)	0.61	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 35E Effect of different factors on IgG responses to MSP2(3D7) in the May 2004 cross sectional bleed.

	MSP2(3D7) May 2004		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.06 (0.04-0.08)	**<0.001**	279
Parasitemia	0.71 (0.49-0.93)	**<0.001**	280
Alpha-thal HET	-0.09 (-0.22-0.05)	0.202	261
Alpha-thal HOM	-0.2 (-0.37--0.03)	**0.02**	
Sickle trait	-0.11 (-0.28-0.07)	0.247	266
Prior parasitemia	0.11 (-0.02-0.24)	0.105	254
Prior episode	-	-	
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.05 (0.03-0.08)	**<0.001**	239
Parasitemia	0.51 (0.27-0.75)	**<0.001**	
Alpha-thal HET	-0.06 (-0.18-0.07)	0.371	
Alpha-thal HOM	-0.12 (-0.28-0.04)	0.144	
Sickle trait	-0.08 (-0.24-0.08)	0.321	
Prior parasitemia	0.06 (-0.07-0.18)	0.385	
Parasite negative[7]			
Age (years)	0.04 (0.02-0.07)	**<0.001**	224
Alpha-thal HET	-0.07 (-0.18-0.05)	0.251	
Alpha-thal HOM	-0.11 (-0.26-0.03)	0.114	
Sickle trait	-0.06 (-0.21-0.08)	0.386	
Prior parasitemia	0.16 (0.03-0.29)	**0.015**	
Parasite positive[8]			
Age (years)	0 (-0.38-0.39)	0.979	15
Alpha-thal HET	-0.38 (-2.03-1.28)	0.618	
Alpha-thal HOM	-1.24 (-4-1.52)	0.337	
Sickle trait	-1 (-3.38-1.38)	0.367	
Prior parasitemia	-0.49 (-1.59-0.6)	0.333	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.05 (0.03-0.08)	**<0.001**	239
Parasitemia	0.53 (0.29-0.76)	**<0.001**	
Alpha-thal HET	-0.06 (-0.19-0.06)	0.326	
Alpha-thal HOM	-0.12 (-0.28-0.04)	0.133	
Sickle trait	-0.08 (-0.24-0.08)	0.323	
Prior episode	-	-	
Parasite negative[7]			
Age (years)	0.05 (0.03-0.07)	**<0.001**	224
Alpha-thal HET	-0.07 (-0.19-0.04)	0.202	
Alpha-thal HOM	-0.12 (-0.26-0.03)	0.114	
Sickle trait	-0.06 (-0.21-0.09)	0.433	
Prior episode	-	-	
Parasite positive[8]			
Age (years)	0.11 (-0.18-0.41)	0.407	15
Alpha-thal HET	-0.13 (-1.67-1.41)	0.855	
Alpha-thal HOM	-0.87 (-3.47-1.73)	0.474	
Sickle trait	-0.39 (-2.32-1.55)	0.665	
Prior episode	-	-	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 35F Effect of different factors on IgG responses to MSP2(3D7) in the October 2004 cross sectional bleed.

	MSP2(3D7) Oct 2004		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.05 (0.03-0.07)	**<0.001**	273
Parasitemia	1.08 (0.86-1.31)	**<0.001**	273
Alpha-thal HET	-0.07 (-0.18-0.05)	0.246	268
Alpha-thal HOM	-0.14 (-0.29-0)	0.058	
Sickle trait	0.02 (-0.12-0.17)	0.752	270
Prior parasitemia	0 (-0.28-0.28)	0.986	235
Prior episode	-	-	
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.06 (0.04-0.08)	**<0.001**	216
Parasitemia	1.25 (0.98-1.52)	**<0.001**	
Alpha-thal HET	-0.04 (-0.14-0.07)	0.489	
Alpha-thal HOM	-0.11 (-0.24-0.02)	0.105	
Sickle trait	-0.12 (-0.25-0.02)	0.087	
Prior parasitemia	0.04 (-0.2-0.27)	0.761	
Parasite negative[7]			
Age (years)	0.05 (0.04-0.07)	**<0.001**	209
Alpha-thal HET	-0.06 (-0.16-0.04)	0.207	
Alpha-thal HOM	-0.12 (-0.24-0.01)	0.063	
Sickle trait	-0.01 (-0.14-0.11)	0.855	
Prior parasitemia	0.04 (-0.17-0.26)	0.709	
Parasite positive[8]			
Age (years)	0.07 (-0.03-0.17)	0.088	7
Alpha-thal HET	-0.04 (-0.64-0.56)	0.807	
Alpha-thal HOM	0.7 (-0.07-1.47)	0.06	
Sickle trait	-1.81 (-2.38--1.24)	**0.005**	
Prior parasitemia	-	-	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.06 (0.04-0.08)	**<0.001**	216
Parasitemia	1.25 (0.98-1.51)	**<0.001**	
Alpha-thal HET	-0.04 (-0.14-0.07)	0.503	
Alpha-thal HOM	-0.11 (-0.24-0.02)	0.11	
Sickle trait	-0.12 (-0.25-0.02)	0.086	
Prior episode	-	-	
Parasite negative[7]			
Age (years)	0.05 (0.04-0.07)	**<0.001**	209
Alpha-thal HET	-0.06 (-0.16-0.04)	0.216	
Alpha-thal HOM	-0.12 (-0.24-0.01)	0.067	
Sickle trait	-0.01 (-0.14-0.11)	0.854	
Prior episode	-	-	
Parasite positive[8]			
Age (years)	0.07 (-0.03-0.17)	0.088	7
Alpha-thal HET	-0.04 (-0.64-0.56)	0.807	
Alpha-thal HOM	0.7 (-0.07-1.47)	0.06	
Sickle trait	-1.81 (-2.38--1.24)	**0.005**	
Prior episode	-	-	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 36A Effect of different factors on IgG responses to MSP2(FC27) in the May 2002 cross sectional bleed.

	MSP2(FC27) May 2002		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.07 (0.04-0.1)	**<0.001**	298
Parasitemia	0.51 (0.33-0.69)	**<0.001**	298
Alpha-thal HET	0.07 (-0.09-0.24)	0.397	297
Alpha-thal HOM	-0.02 (-0.23-0.2)	0.882	
Sickle trait	-0.15 (-0.36-0.06)	0.149	299
Prior parasitemia	0.25 (0.17-0.33)	**<0.001**	304
Prior episode	0.22 (0.08-0.35)	**0.002**	304
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.04 (0-0.07)	**0.026**	289
Parasitemia	0.39 (0.2-0.57)	**<0.001**	
Alpha-thal HET	0.1 (-0.06-0.25)	0.215	
Alpha-thal HOM	0.07 (-0.13-0.27)	0.478	
Sickle trait	-0.14 (-0.34-0.05)	0.156	
Prior parasitemia	0.17 (0.09-0.26)	**<0.001**	
Parasite negative[7]			
Age (years)	0.03 (0-0.06)	0.078	239
Alpha-thal HET	0.06 (-0.09-0.22)	0.431	
Alpha-thal HOM	0.02 (-0.18-0.21)	0.873	
Sickle trait	-0.12 (-0.32-0.09)	0.259	
Prior parasitemia	0.2 (0.11-0.29)	**<0.001**	
Parasite positive[8]			
Age (years)	0.06 (-0.08-0.2)	0.409	50
Alpha-thal HET	0.19 (-0.36-0.74)	0.486	
Alpha-thal HOM	0.43 (-0.49-1.35)	0.348	
Sickle trait	-0.23 (-0.84-0.37)	0.441	
Prior parasitemia	0.13 (-0.12-0.38)	0.302	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.05 (0.02-0.08)	**0.004**	289
Parasitemia	0.44 (0.25-0.63)	**<0.001**	
Alpha-thal HET	0.07 (-0.09-0.23)	0.383	
Alpha-thal HOM	0.06 (-0.14-0.27)	0.553	
Sickle trait	-0.15 (-0.35-0.05)	0.138	
Prior episode	0.11 (-0.02-0.25)	0.102	
Parasite negative[7]			
Age (years)	0.04 (0.01-0.08)	**0.007**	239
Alpha-thal HET	0.05 (-0.11-0.21)	0.52	
Alpha-thal HOM	0.01 (-0.19-0.21)	0.91	
Sickle trait	-0.14 (-0.35-0.07)	0.182	
Prior episode	0.18 (0.03-0.33)	**0.017**	
Parasite positive[8]			
Age (years)	0.04 (-0.1-0.19)	0.544	50
Alpha-thal HET	0.09 (-0.45-0.64)	0.725	
Alpha-thal HOM	0.38 (-0.58-1.33)	0.433	
Sickle trait	-0.23 (-0.86-0.4)	0.461	
Prior episode	-0.02 (-0.42-0.37)	0.909	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 36B Effect of different factors on IgG responses to MSP2(FC27) in the October 2002 cross sectional bleed.

	MSP2(FC27) Oct 2002		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.06 (0.04-0.09)	**<0.001**	291
Parasitemia	0.84 (0.65-1.03)	**<0.001**	291
Alpha-thal HET	0.03 (-0.1-0.15)	0.655	284
Alpha-thal HOM	-0.17 (-0.32--0.01)	0.04	
Sickle trait	-0.09 (-0.26-0.08)	0.296	292
Prior parasitemia	0.17 (0.06-0.28)	**0.002**	254
Prior episode	0.22 (0.08-0.36)	**0.002**	254
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.06 (0.03-0.08)	**<0.001**	251
Parasitemia	0.7 (0.5-0.9)	**<0.001**	
Alpha-thal HET	0.02 (-0.1-0.14)	0.724	
Alpha-thal HOM	-0.11 (-0.27-0.04)	0.138	
Sickle trait	-0.08 (-0.24-0.09)	0.353	
Prior parasitemia	0.13 (0.04-0.23)	**0.007**	
Parasite negative[7]			
Age (years)	0.05 (0.03-0.07)	**<0.001**	232
Alpha-thal HET	0.03 (-0.08-0.14)	0.577	
Alpha-thal HOM	-0.07 (-0.21-0.07)	0.304	
Sickle trait	-0.05 (-0.2-0.1)	0.494	
Prior parasitemia	0.14 (0.05-0.23)	**0.002**	
Parasite positive[8]			
Age (years)	0.27 (-0.01-0.55)	0.059	19
Alpha-thal HET	-0.61 (-1.8-0.59)	0.293	
Alpha-thal HOM	-1.46 (-2.92-0)	**0.05**	
Sickle trait	-0.65 (-1.95-0.66)	0.303	
Prior parasitemia	0.15 (-0.63-0.93)	0.684	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.05 (0.03-0.08)	**<0.001**	251
Parasitemia	0.72 (0.52-0.91)	**<0.001**	
Alpha-thal HET	0.02 (-0.1-0.14)	0.767	
Alpha-thal HOM	-0.11 (-0.27-0.04)	0.136	
Sickle trait	-0.08 (-0.25-0.08)	0.307	
Prior episode	0.18 (0.06-0.3)	**0.004**	
Parasite negative[7]			
Age (years)	0.05 (0.03-0.07)	**<0.001**	232
Alpha-thal HET	0.02 (-0.09-0.13)	0.67	
Alpha-thal HOM	-0.07 (-0.21-0.06)	0.293	
Sickle trait	-0.06 (-0.21-0.09)	0.446	
Prior episode	0.21 (0.09-0.32)	**<0.001**	
Parasite positive[8]			
Age (years)	0.25 (-0.01-0.52)	0.061	19
Alpha-thal HET	-0.52 (-1.63-0.59)	0.328	
Alpha-thal HOM	-1.46 (-2.93-0.01)	0.051	
Sickle trait	-0.69 (-1.98-0.59)	0.265	
Prior episode	0.06 (-0.77-0.89)	0.884	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 36C Effect of different factors on IgG responses to MSP2(FC27) in the May 2003 cross sectional bleed.

	MSP2(FC27) May 2003		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.07 (0.05-0.1)	**<0.001**	285
Parasitemia	0.71 (0.54-0.87)	**<0.001**	285
Alpha-thal HET	-0.07 (-0.21-0.07)	0.354	279
Alpha-thal HOM	-0.22 (-0.4--0.04)	**0.016**	
Sickle trait	-0.18 (-0.37-0.01)	0.064	283
Prior parasitemia	0.04 (-0.01-0.09)	0.119	253
Prior episode	0.03 (-0.04-0.1)	0.429	253
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.06 (0.04-0.09)	**<0.001**	247
Parasitemia	0.62 (0.45-0.8)	**<0.001**	
Alpha-thal HET	-0.06 (-0.19-0.07)	0.372	
Alpha-thal HOM	-0.13 (-0.3-0.04)	0.138	
Sickle trait	-0.16 (-0.34-0.02)	0.088	
Prior parasitemia	0.02 (-0.03-0.06)	0.485	
Parasite negative[7]			
Age (years)	0.04 (0.02-0.07)	**<0.001**	213
Alpha-thal HET	-0.04 (-0.15-0.08)	0.561	
Alpha-thal HOM	-0.11 (-0.26-0.03)	0.126	
Sickle trait	-0.09 (-0.24-0.07)	0.267	
Prior parasitemia	0.02 (-0.02-0.06)	0.333	
Parasite positive[8]			
Age (years)	0.16 (0.03-0.3)	**0.016**	34
Alpha-thal HET	-0.1 (-0.72-0.53)	0.753	
Alpha-thal HOM	-0.18 (-1.33-0.97)	0.752	
Sickle trait	-0.5 (-1.6-0.6)	0.359	
Prior parasitemia	0.03 (-0.22-0.29)	0.786	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.06 (0.04-0.09)	**<0.001**	247
Parasitemia	0.63 (0.46-0.8)	**<0.001**	
Alpha-thal HET	-0.06 (-0.19-0.07)	0.376	
Alpha-thal HOM	-0.13 (-0.3-0.04)	0.134	
Sickle trait	-0.16 (-0.34-0.02)	0.083	
Prior episode	0.02 (-0.05-0.08)	0.617	
Parasite negative[7]			
Age (years)	0.04 (0.02-0.07)	**<0.001**	213
Alpha-thal HET	-0.04 (-0.15-0.08)	0.56	
Alpha-thal HOM	-0.11 (-0.26-0.03)	0.123	
Sickle trait	-0.09 (-0.24-0.07)	0.266	
Prior episode	0.03 (-0.02-0.08)	0.215	
Parasite positive[8]			
Age (years)	0.17 (0.04-0.3)	**0.013**	34
Alpha-thal HET	-0.08 (-0.69-0.53)	0.789	
Alpha-thal HOM	0.04 (-1.17-1.24)	0.952	
Sickle trait	-0.58 (-1.69-0.53)	0.29	
Prior episode	-0.19 (-0.64-0.27)	0.416	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 36D Effect of different factors on IgG responses to MSP2(FC27) in the October 2003 cross sectional bleed.

	MSP2(FC27) Oct 2003		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.08 (0.05-0.1)	**<0.001**	294
Parasitemia	0.79 (0.63-0.95)	**<0.001**	293
Alpha-thal HET	-0.04 (-0.18-0.11)	0.61	285
Alpha-thal HOM	-0.23 (-0.42--0.04)	**0.015**	
Sickle trait	-0.14 (-0.34-0.05)	0.147	284
Prior parasitemia	0.13 (0.05-0.21)	**0.001**	261
Prior episode	0.14 (0.03-0.26)	**0.015**	261
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.06 (0.04-0.09)	**<0.001**	248
Parasitemia	0.68 (0.51-0.86)	**<0.001**	
Alpha-thal HET	-0.07 (-0.2-0.07)	0.323	
Alpha-thal HOM	-0.15 (-0.33-0.02)	0.081	
Sickle trait	-0.17 (-0.34-0.01)	0.063	
Prior parasitemia	0.08 (0.02-0.15)	**0.017**	
Parasite negative[7]			
Age (years)	0.04 (0.02-0.07)	**<0.001**	212
Alpha-thal HET	0.02 (-0.1-0.14)	0.731	
Alpha-thal HOM	-0.06 (-0.21-0.09)	0.434	
Sickle trait	-0.12 (-0.28-0.03)	0.126	
Prior parasitemia	0.18 (0.12-0.24)	**<0.001**	
Parasite positive[8]			
Age (years)	0.15 (0.01-0.29)	**0.04**	36
Alpha-thal HET	-0.23 (-0.76-0.3)	0.388	
Alpha-thal HOM	-0.07 (-1.14-0.99)	0.891	
Sickle trait	-0.63 (-1.31-0.05)	0.067	
Prior parasitemia	-0.27 (-0.56-0.02)	0.065	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.06 (0.04-0.09)	**<0.001**	248
Parasitemia	0.71 (0.54-0.88)	**<0.001**	
Alpha-thal HET	-0.07 (-0.2-0.07)	0.349	
Alpha-thal HOM	-0.14 (-0.31-0.04)	0.122	
Sickle trait	-0.16 (-0.34-0.01)	0.073	
Prior episode	0.11 (0.01-0.21)	**0.031**	
Parasite negative[7]			
Age (years)	0.05 (0.02-0.07)	**<0.001**	212
Alpha-thal HET	0.01 (-0.12-0.14)	0.875	
Alpha-thal HOM	-0.05 (-0.21-0.1)	0.501	
Sickle trait	-0.1 (-0.26-0.06)	0.227	
Prior episode	0.18 (0.1-0.27)	**<0.001**	
Parasite positive[8]			
Age (years)	0.18 (0.04-0.32)	**0.015**	36
Alpha-thal HET	-0.33 (-0.86-0.2)	0.215	
Alpha-thal HOM	-0.4 (-1.46-0.65)	0.442	
Sickle trait	-0.51 (-1.2-0.19)	0.147	
Prior episode	-0.24 (-0.67-0.19)	0.264	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 36E Effect of different factors on IgG responses to MSP2(FC27) in the May 2004 cross sectional bleed.

	MSP2(FC27) May 2004		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.05 (0.03-0.07)	**<0.001**	279
Parasitemia	0.71 (0.5-0.91)	**<0.001**	280
Alpha-thal HET	-0.07 (-0.2-0.06)	0.274	261
Alpha-thal HOM	-0.21 (-0.37--0.04)	**0.012**	
Sickle trait	-0.13 (-0.3-0.03)	0.117	266
Prior parasitemia	0.23 (0.11-0.35)	**<0.001**	254
Prior episode	-	-	
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.05 (0.02-0.07)	**<0.001**	239
Parasitemia	0.51 (0.28-0.73)	**<0.001**	
Alpha-thal HET	-0.04 (-0.16-0.08)	0.485	
Alpha-thal HOM	-0.12 (-0.27-0.03)	0.112	
Sickle trait	-0.12 (-0.27-0.03)	0.128	
Prior parasitemia	0.16 (0.04-0.28)	**0.01**	
Parasite negative[7]			
Age (years)	0.04 (0.02-0.06)	**<0.001**	224
Alpha-thal HET	-0.08 (-0.19-0.04)	0.191	
Alpha-thal HOM	-0.13 (-0.27-0.01)	0.063	
Sickle trait	-0.08 (-0.22-0.07)	0.29	
Prior parasitemia	0.14 (0.01-0.26)	**0.032**	
Parasite positive[8]			
Age (years)	0.2 (-0.12-0.53)	0.189	15
Alpha-thal HET	0.27 (-1.12-1.66)	0.668	
Alpha-thal HOM	-0.4 (-2.72-1.92)	0.706	
Sickle trait	-0.07 (-2.07-1.93)	0.939	
Prior parasitemia	0.55 (-0.37-1.46)	0.211	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.05 (0.03-0.07)	**<0.001**	239
Parasitemia	0.55 (0.33-0.78)	**<0.001**	
Alpha-thal HET	-0.06 (-0.18-0.06)	0.349	
Alpha-thal HOM	-0.13 (-0.28-0.02)	0.092	
Sickle trait	-0.12 (-0.28-0.04)	0.135	
Prior episode	-	-	
Parasite negative[7]			
Age (years)	0.05 (0.02-0.07)	**<0.001**	224
Alpha-thal HET	-0.08 (-0.2-0.03)	0.155	
Alpha-thal HOM	-0.13 (-0.27-0.01)	0.063	
Sickle trait	-0.07 (-0.22-0.07)	0.325	
Prior episode	-	-	
Parasite positive[8]			
Age (years)	0.08 (-0.17-0.34)	0.494	15
Alpha-thal HET	0 (-1.34-1.34)	0.999	
Alpha-thal HOM	-0.81 (-3.07-1.46)	0.447	
Sickle trait	-0.75 (-2.43-0.94)	0.348	
Prior episode	-	-	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 36F Effect of different factors on IgG responses to MSP2(FC27) in the October 2004 cross sectional bleed.

	MSP2(FC27) Oct 2004		
	β (95% CI)[2]	*P*-value[3]	N[4]
Univariate[5]			
Age (years)	0.04 (0.03-0.06)	**<0.001**	273
Parasitemia	0.55 (0.33-0.77)	**<0.001**	273
Alpha-thal HET	-0.02 (-0.11-0.08)	0.762	268
Alpha-thal HOM	-0.1 (-0.23-0.02)	0.11	
Sickle trait	-0.06 (-0.19-0.07)	0.345	270
Prior parasitemia	0.05 (-0.21-0.3)	0.725	235
Prior episode	-	-	
Multivariate (previous parasitemia)[6]			
All samples			
Age (years)	0.05 (0.03-0.07)	**<0.001**	216
Parasitemia	0.64 (0.36-0.92)	**<0.001**	
Alpha-thal HET	-0.02 (-0.13-0.09)	0.732	
Alpha-thal HOM	-0.11 (-0.25-0.03)	0.127	
Sickle trait	-0.11 (-0.25-0.03)	0.112	
Prior parasitemia	0.07 (-0.18-0.31)	0.596	
Parasite negative[7]			
Age (years)	0.05 (0.03-0.07)	**<0.001**	209
Alpha-thal HET	-0.06 (-0.17-0.05)	0.289	
Alpha-thal HOM	-0.13 (-0.27-0.01)	0.065	
Sickle trait	-0.08 (-0.22-0.06)	0.279	
Prior parasitemia	0.07 (-0.16-0.31)	0.54	
Parasite positive[8]			
Age (years)	0.04 (-0.18-0.26)	0.528	7
Alpha-thal HET	1.33 (-0.05-2.72)	0.053	
Alpha-thal HOM	0.07 (-1.71-1.84)	0.883	
Sickle trait	0 (-1.31-1.31)	0.995	
Prior parasitemia	-	-	
Multivariate (previous episodes)[6]			
All samples			
Age (years)	0.05 (0.03-0.07)	**<0.001**	216
Parasitemia	0.64 (0.36-0.91)	**<0.001**	
Alpha-thal HET	-0.02 (-0.13-0.09)	0.766	
Alpha-thal HOM	-0.1 (-0.24-0.03)	0.141	
Sickle trait	-0.11 (-0.25-0.03)	0.111	
Prior episode	-	-	
Parasite negative[7]			
Age (years)	0.05 (0.03-0.07)	**<0.001**	209
Alpha-thal HET	-0.06 (-0.17-0.05)	0.312	
Alpha-thal HOM	-0.12 (-0.26-0.01)	0.074	
Sickle trait	-0.08 (-0.22-0.06)	0.277	
Prior episode	-	-	
Parasite positive[8]			
Age (years)	0.04 (-0.18-0.26)	0.528	7
Alpha-thal HET	1.33 (-0.05-2.72)	0.053	
Alpha-thal HOM	0.07 (-1.71-1.84)	0.883	
Sickle trait	0 (-1.31-1.31)	0.995	
Prior episode	-	-	

Notes

[1] Antigen tested at indicated cross sectional bleed.

[2] Linear regression β coefficients with 95% confidence intervals

[3] *P*-value for indicated factor (*p*-values ≤ 0.05 indicated in bold).

[4] Number of samples included in the model.

[5] Univariate linear regression.

[6] Multivariable linear regression with inclusion of either recorded episodes of parasitemia or malaria in the 6 months prior to sampling.

[7] Stratified multivariable linear regression, parasite negative samples only.

[8] Stratified multivariable linear regression, parasite positive samples only.

Table 37 Correlations between IgG responses to AMA1(HB3) at each cross sectional bleed.

	IgG responses to AMA1 (HB3)					
	May 2002[1]	October 2002[1]	May 2003[1]	October 2003[1]	May 2004[1]	October 2004[1]
May 2002[1]	-	-	-	-	-	-
October 2002[1]	0.71* (308)	-	-	-	-	-
May 2003[1]	0.57* (263)	0.48* (294)	-	-	-	-
October 2003[1]	0.57* (249)	0.51* (259)	0.58* (293)	-	-	-
May 2004[1]	0.56* (231)	0.52* (242)	0.52* (259)	0.72* (278)	-	-
October 2004[1]	0.57* (226)	0.48* (236)	0.56* (255)	0.74* (274)	0.74* (277)	-

Notes

Spearman rank correlation coefficients for IgG responses.

Number of samples in each comparison indicated in brackets.

[1] Cross sectional bleed.

* *P*-value <0.001

Table 38 Correlations between IgG responses to AMA1(3D7) at each cross sectional bleed.

	IgG responses to AMA1 (3D7)					
	May 2002[1]	October 2002[1]	May 2003[1]	October 2003[1]	May 2004[1]	October 2004[1]
May 2002[1]	-	-	-	-	-	-
October 2002[1]	0.72* (308)	-	-	-	-	-
May 2003[1]	0.6* (263)	0.54* (294)	-	-	-	-
October 2003[1]	0.62* (249)	0.51* (259)	0.56* (293)	-	-	-
May 2004[1]	0.63* (231)	0.56* (242)	0.56* (259)	0.72* (278)	-	-
October 2004[1]	0.6* (226)	0.48* (236)	0.55* (255)	0.7* (274)	0.73* (277)	-

Notes

Spearman rank correlation coefficients for IgG responses.

Number of samples in each comparison indicated in brackets.

[1] Cross sectional bleed.

* *P*-value <0.001

Table 39 Correlations between IgG responses to MSP2(FC27) at each cross sectional bleed.

	IgG responses to MSP2 (FC27)					
	May 2002[1]	October 2002[1]	May 2003[1]	October 2003[1]	May 2004[1]	October 2004[1]
May 2002[1]	-	-	-	-	-	-
October 2002[1]	0.68* (308)	-	-	-	-	-
May 2003[1]	0.47* (263)	0.62* (294)	-	-	-	-
October 2003[1]	0.5* (249)	0.54* (259)	0.56* (293)	-	-	-
May 2004[1]	0.54* (231)	0.56* (242)	0.58* (259)	0.67* (278)	-	-
October 2004[1]	0.52* (226)	0.62* (236)	0.52* (255)	0.6* (274)	0.73* (277)	-

Notes

Spearman rank correlation coefficients for IgG responses.

Number of samples in each comparison indicated in brackets.

[1] Cross sectional bleed.

* *P*-value <0.001

APPENDIX 2

The following appendix includes all results not included in **Chapter 4** but mentioned in the text.

Tables 1–3: The rate of decay of antibody responses to AMA1 and MSP2 between May 2004 and October 2004.

Figures 1–4: Longitudinal antibody responses to AMA1 and MSP2.

Tables 4–5: Antibody responses in aparasitemic and parasitemic individuals to AMA1 and MSP2 in October 2002 categorised by their incidence of concurrent parasitemia at sampling throughout the study.

Table 1 Differences in the rate of anti-AMA1(HB3) IgG decay between May and October 2004

	Anti-AMA1(HB3) IgG		
	β (95% CI) [ǂ]	*P*-value[Δ]	N[ε]
Sample time[1]	-0.2 (-0.26, -0.14)	**<0.001**	91
Age 0-2 years	-0.46 (-0.76, -0.17)	**0.002**	10
Age 3-5 years	-0.2 (-0.31, -0.09)	**<0.001**	27
Age 6-9 years	-0.16 (-0.22, -0.1)	**<0.001**	54
Compared to 0-2 year olds			
... Age 3-5 years	0.28 (-0.18, 0.74)	0.233	91
... Age 6-9 years	0.54 (0.11, 0.97)	**0.014**	
HbAA (Normal)	-0.23 (-0.29, -0.16)	**<0.001**	77
HbAS (Sickle trait)	-0.04 (-0.24, 0.16)	0.685	11
Compared to HbAA children			
..... HbAS	-0.26 (-0.67, 0.16)	0.221	88
Alpha-thalassemia NORM[2]	-0.23 (-0.31, -0.15)	**<0.001**	32
Alpha-thalassemia HET[2]	-0.23 (-0.33, -0.12)	**<0.001**	42
Alpha-thalassemia HOM[2]	-0.04 (-0.19, 0.11)	0.584	12
Compared to normal children			
.... Alpha-thalassemia HET	-0.09 (-0.38, 0.21)	0.578	86
.... Alpha-thalassemia HOM	-0.34 (-0.78, 0.09)	0.119	

Notes

Univariate generalized estimating equation (GEE) with stationary correlation structure.

[ǂ] GEE regression coefficients indicating reduction/increase in the rate of antibody decay between May and October 2004.

[Δ] *P*-values ≤ 0.05 indicated in bold type.

[ε] Number of samples included in the model.

[1] Change in IgG response between May and October 2004.

[2] Alpha-thalassemia genotypes: NORM = normal, HET = heterozygous, HOM = homozygous.

Table 2 Differences in the rate of anti-AMA1(3D7) IgG decay between May and October 2004

	Anti-AMA1(3D7) IgG		
	β (95% CI)[ǂ]	*P*-value[Δ]	N[ε]
Sample time[1]	-0.19 (-0.25, -0.13)	**<0.001**	81
Age 0-2 years	-0.36 (-0.64, -0.08)	**0.011**	10
Age 3-5 years	-0.22 (-0.32, -0.12)	**<0.001**	21
Age 6-9 years	-0.15 (-0.21, -0.09)	**<0.001**	50
Compared to 0-2 year olds			
... Age 3-5 years	0.3 (-0.16, 0.76)	0.203	81
... Age 6-9 years	0.53 (0.11, 0.94)	**0.013**	
HbAA (Normal)	-0.21 (-0.27, -0.14)	**<0.001**	70
HbAS (Sickle trait)	-0.07 (-0.23, 0.09)	0.412	9
Compared to HbAA children			
..... HbAS	-0.29 (-0.72, 0.15)	0.197	79
Alpha-thalassemia NORM[2]	-0.17 (-0.25, -0.1)	**<0.001**	31
Alpha-thalassemia HET[2]	-0.23 (-0.34, -0.12)	**<0.001**	37
Alpha-thalassemia HOM[2]	-0.08 (-0.15, -0.02)	**0.013**	9
Compared to normal children			
.... Alpha-thalassemia HET	0.1 (-0.2, 0.4)	0.521	77
.... Alpha-thalassemia HOM	-0.15 (-0.62, 0.31)	0.520	

Notes

Univariate generalized estimating equation (GEE) with stationary correlation structure.

[ǂ]GEE regression coefficients indicating reduction/increase in the rate of antibody decay between May and October 2004.

[Δ]*P*-values ≤ 0.05 indicated in bold type.

[ε]Number of samples included in the model.

[1]Change in IgG response between May and October 2004.

[2]Alpha-thalassemia genotypes: NORM = normal, HET = heterozygous, HOM = homozygous.

Table 3 Differences in the rate of anti-MSP2(FC27) IgG decay between May and October 2004

	Anti-MSP2(FC27) IgG		
	β (95% CI)[ǂ]	*P*-value[Δ]	N[ε]
Sample time[1]	-0.36 (-0.49, -0.23)	**<0.001**	24
Age 0-2 years	-0.85 (-1.41, -0.29)	**0.003**	3
Age 3-5 years	-0.29 (-0.42, -0.15)	**<0.001**	6
Age 6-9 years	-0.29 (-0.4, -0.18)	**<0.001**	15
Compared to 0-2 year olds			
... Age 3-5 years	0.15 (-0.77, 1.06)	0.755	24
... Age 6-9 years	0.62 (-0.2, 1.44)	0.138	
HbAA (Normal)	-0.35 (-0.48, -0.21)	**<0.001**	22
HbAS (Sickle trait)	-0.52 (-0.57, -0.47)	**<0.001**	2
Compared to HbAA children			
..... HbAS	-0.55 (-1.55, 0.46)	0.286	24
Alpha-thalassemia NORM[2]	-0.46 (-0.7, -0.21)	**<0.001**	10
Alpha-thalassemia HET[2]	-0.26 (-0.39, -0.13)	**<0.001**	10
Alpha-thalassemia HOM[2]	-0.25 (-0.45, -0.06)	**0.010**	3
Compared to normal children			
.... Alpha-thalassemia HET	0.11 (-0.47, 0.7)	0.707	23
.... Alpha-thalassemia HOM	-0.72 (-1.58, 0.14)	0.099	

Notes

Univariate generalized estimating equation (GEE) with stationary correlation structure.

[ǂ]GEE regression coefficients indicating reduction/increase in the rate of antibody decay between May and October 2004.

[Δ]*P*-values ≤ 0.05 indicated in bold type.

[ε]Number of samples included in the model.

[1]Change in IgG response between May and October 2004.

[2]Alpha-thalassemia genotypes: NORM = normal, HET = heterozygous, HOM = homozygous.

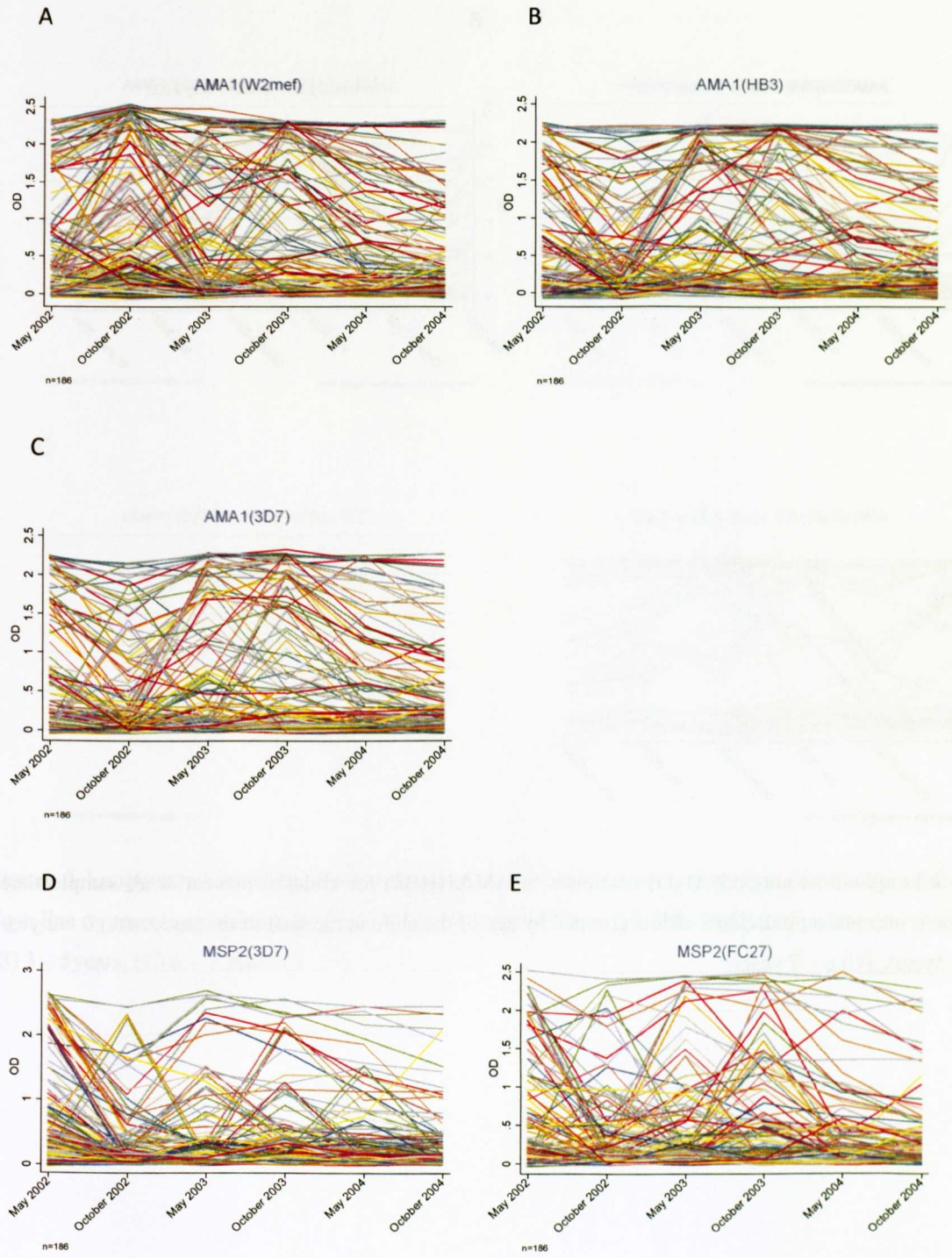

Figure 1 Longitudinal antibody (IgG) responses to merozoite antigens for children present at all six sample times. Each line represents an individual. (A) AMA1(W2mef), (B) AMA1(HB3), (C) AMA1(3D7), (D) MSP2(3D7), (E) MSP2(FC27).

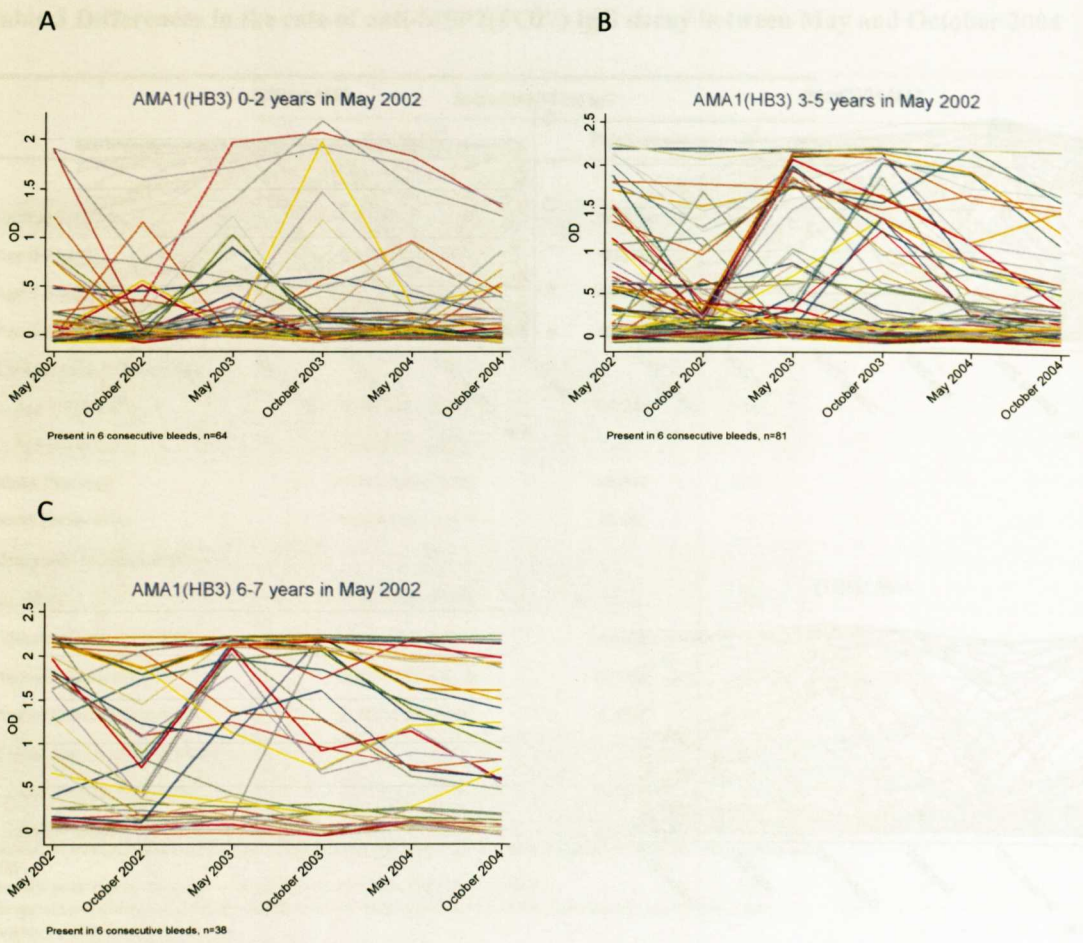

Figure 2 Longitudinal antibody (IgG) responses to AMA1(HB3) for children present at all sample times. Each line represents an individual. Plots grouped by age of the child at the start of the study. (A) 0 – 2 years, (B) 3 – 5years, (C) 6 – 7 years.

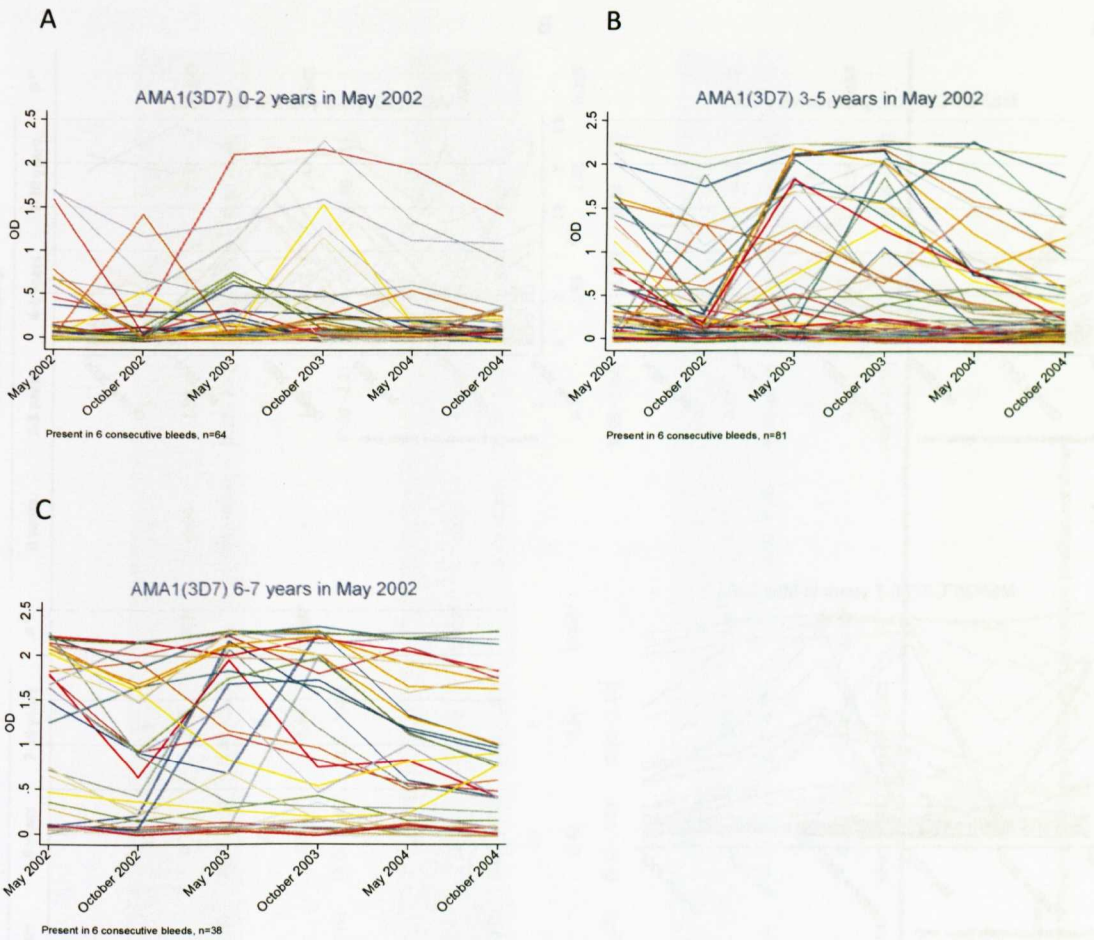

Figure 3 Longitudinal antibody (IgG) responses to AMA1(3D7) in children present at all sample times. Each line represents an individual. Plots grouped by age of the child at the start of the study. (A) 0 – 2 years, (B) 3 – 5years, (C) 6 – 7 years.

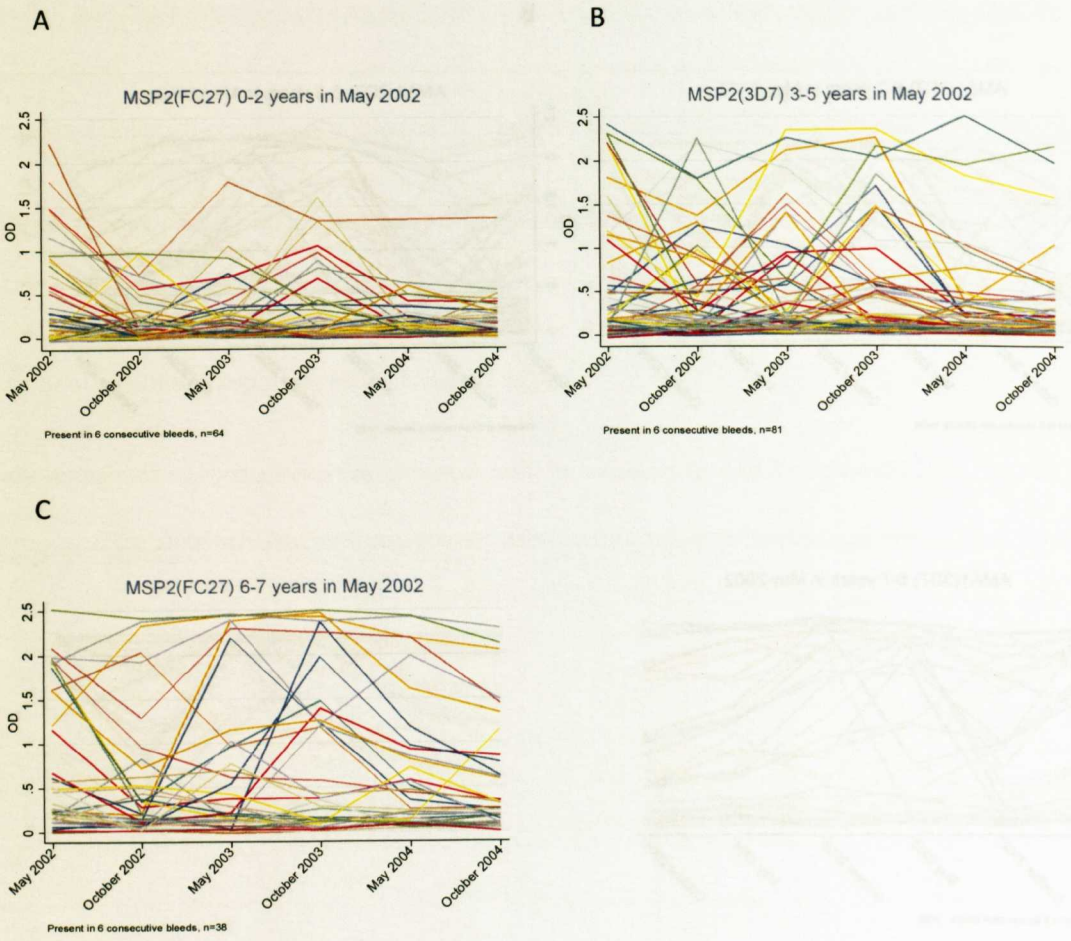

Figure 4 Longitudinal antibody (IgG) responses to MSP2(FC27) in children present at all sample times. Each line represents an individual. Plots grouped by age of the child at the start of the study. (A) 0 – 2 years, (B) 3 – 5years, (C) 6 – 7 years.

Table 4 IgG responses in aparasitemic and parasitemic individuals to AMA1 variants in October 2002 categorised by their incidence of concurrent parasitemia at sampling throughout the study

Concurrent parasitemia[Δ]		All Samples					Aparasitemic at sampling[1]					Parasitemic at sampling[2]				
		0 years	1-3 years	4-6 years	7-10 years	P^3	0 years	1-3 years	4-6 years	7-10 years	P^3	0 years	1-3 years	4-6 years	7-10 years	P^3
AMA1(W2mef)																
Once[Δ]	N	6	24	29	11		5	22	27	9		1	2	2	2	
	Median	0.05	0.42	0.49[‡]	0.49[‡]	**0.012***	0.08	0.39	0.71	2.17	**0.008***	-0.04	1.80	0.11[‡]	0.37	0.185
	IQR	(0.03 - 0.13)	(0.12 - 1.13)	(0.12 - 1.23)	(0.39 - 2.29)		(0.03 - 0.13)	(0.08 - 0.76)	(0.17 - 1.3)	(1.41 - 2.29)		(-0.04 - -0.04)	(1.27 - 2.32)	(0.1 - 0.12)	(0.09 - 0.66)	
Recurrent[Δ]	N	1	7	17	9		1	4	10	6		-	3	7	3	
	Median	0.07	0.39	1.54[‡]	1.54[‡]	**0.010***	0.07	0.18	1.38	2.42	**0.038***	-	1.13	2.00[‡]	2.47	0.341
	IQR	(0.07 - 0.07)	(-0.04 - 1.13)	(0.48 - 2.36)	(1.6 - 2.47)		(0.07 - 0.07)	(-0.04 - 0.44)	(0.22 - 2.45)	(1.6 - 2.47)		-	(0.08 - 2.2)	(1.16 - 2.31)	(0.86 - 2.5)	
AMA1(HB3)																
Once[Δ]	N	6	24	29	11		5	22	27	9		1	2	2	2	
	Median	0.05	0.12	0.13	0.13[‡]	0.067	0.05	0.09	0.13	0.77	0.052*	0.05	1.08	0.52	0.10	0.426
	IQR	(0.02 - 0.05)	(0.04 - 0.43)	(0.06 - 0.88)	(0.09 - 1.22)		(0.02 - 0.05)	(0.04 - 0.31)	(0.06 - 0.88)	(0.25 - 1.22)		(0.05 - 0.05)	(0.56 - 1.59)	(0.02 - 1.02)	(0.09 - 0.1)	
Recurrent[Δ]	N	1	7	17	9		1	4	10	6		-	3	7	3	
	Median	-0.01	0.08	0.59	0.59[‡]	**0.024***	-0.01	0.03	0.42	1.96	0.096*	-	0.36	0.59	2.05	0.208
	IQR	(-0.01 - -0.01)	(0.03 - 0.4)	(0.17 - 1.52)	(0.44 - 2.13)		(-0.01 - -0.01)	(0.01 - 0.22)	(0.13 - 1.89)	(0.22 - 2.13)		-	(0.08 - 1.04)	(0.2 - 1.23)	(0.44 - 2.11)	
AMA1(3D7)																
Once[Δ]	N	6	24	29	11		5	22	27	9		1	2	2	2	
	Median	0.01	0.05	0.11	0.11[‡]	**0.024***	0.01	0.04	0.11	0.51	**0.010***	0.02	0.84	0.68	0.18	0.522
	IQR	(0 - 0.02)	(0 - 0.2)	(0.02 - 0.6)	(0.05 - 0.91)		(0 - 0.02)	(0 - 0.11)	(0.02 - 0.6)	(0.2 - 0.91)		(0.02 - 0.02)	(0.52 - 1.17)	(0.01 - 1.34)	(0.01 - 0.35)	
Recurrent[Δ]	N	1	7	17	9		1	4	10	6		-	3	7	3	
	Median	-0.01	-0.01	0.34	0.34[‡]	**0.013***	-0.01	-0.02	0.25	1.82	**0.049***	-	0.20	0.34	2.14	0.158
	IQR	(-0.01 - -0.01)	(-0.03 - 0.22)	(0.1 - 1.28)	(0.36 - 2.14)		(-0.01 - -0.01)	(-0.03 - 0.1)	(0.09 - 1.88)	(0.05 - 2.08)		-	(-0.01 - 0.75)	(0.12 - 1.07)	(0.36 - 2.16)	

Notes

[Δ] Incidences of concurrent parasitemia at sampling during the study grouped by 'never' (not shown), 'once', 'more than once/recurrent'.

[1] No observed parasitemia during October 2002 sampling.

[2] Concurrent parasitemia measured during Ocotber 2002 sampling.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[*] *P*-values ≤ 0.05 when comparing IgG levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

[‡] *P*-values ≤ 0.05 when comparing IgG levels between children with recurrent parasitemia and those with a single incidence of parasitemia in the same age group using a Wilcoxon rank sum test (*P*-values not shown).

Table 5 IgG responses in aparasitemic and parasitemic individuals to MSP2 variants in October 2002 categorised by their incidence of concurrent parasitemia at sampling throughout the study

Concurrent parasitemia[Δ]		All Samples					Aparasitemic at sampling[1]					Parasitemic at sampling[2]				
		0 years	1-3 years	4-6 years	7-10 years	P^3	0 years	1-3 years	4-6 years	7-10 years	P^3	0 years	1-3 years	4-6 years	7-10 years	P^3
MSP2(3D7)																
Once[Δ]	N	6	24	29	11	**0.031**	5	22	27	9	0.067	1	2	2	2	0.482
	Median	0.02	0.08	0.11[‡]	0.11[‡]		0.03	0.08[‡]	0.11[‡]	0.09[‡]		0.01	0.96	0.09	0.28	
	IQR	(0.01 - 0.05)	(0.04 - 0.2)	(0.06 - 0.32)	(0.06 - 0.22)		(0.01 - 0.05)	(0.03 - 0.17)	(0.06 - 0.35)	(0.07 - 0.12)		(0.01 - 0.01)	(0.04 - 1.87)	(0.04 - 0.14)	(0.06 - 0.5)	
Recurrent[Δ]	N	1	7	17	9	**0.031***	1	4	10	6	**0.047***	0	3	7	3	0.061*
	Median	0.14	0.02	0.54[‡]	0.54[‡]		0.14	0.02[‡]	0.69[‡]	0.68[‡]		0.00	0.62	0.23	2.43	
	IQR	(0.14 - 0.14)	(0.01 - 0.62)	(0.17 - 0.76)	(0.34 - 1.65)		(0.14 - 0.14)	(0 - 0.05)	(0.12 - 0.78)	(0.13 - 1.51)		(0 - 0)	(0.02 - 0.77)	(0.17 - 0.68)	(1.65 - 2.44)	
MSP2(FC27)																
Once[Δ]	N	6	24	29	11	0.070	5	22	27	9	0.164	1	2	2	2	0.522
	Median	0.02	0.07	0.07[‡]	0.07[‡]		0.02	0.07	0.07	0.13		0.00	0.84	1.01	0.94	
	IQR	(0 - 0.04)	(0.02 - 0.4)	(0.03 - 0.44)	(0.04 - 0.36)		(0.02 - 0.04)	(0.01 - 0.14)	(0.03 - 0.44)	(0.04 - 0.34)		(0 - 0)	(0.71 - 0.97)	(0.02 - 2.01)	(0.08 - 1.81)	
Recurrent[Δ]	N	1	7	17	9	0.162*	1	4	10	6	0.051*	0	3	7	3	0.360
	Median	0.17	0.11	0.42[‡]	0.42[‡]		0.17	0.03	0.30	0.62		0.00	0.78	0.91	2.38	
	IQR	(0.17 - 0.17)	(0 - 0.78)	(0.2 - 1.26)	(0.4 - 2.33)		(0.17 - 0.17)	(-0.01 - 0.09)	(0.17 - 0.51)	(0.12 - 1.29)		(0 - 0)	(0.25 - 1.8)	(0.35 - 1.84)	(0.53 - 2.42)	

Notes

[Δ] Incidences of concurrent parasitemia at sampling during the study grouped by 'never' (not shown), 'once', 'more than once/recurrent'.

[1] No observed parasitemia during October 2002 sampling.

[2] Concurrent parasitemia measured during Ocotber 2002 sampling.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[*] *P*-values ≤ 0.05 when comparing IgG levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

[‡] *P*-values ≤ 0.05 when comparing IgG levels between children with recurrent parasitemia and those with a single incidence of parasitemia in the same age group using a Wilcoxon rank sum test (*P*-values not shown).

APPENDIX 3

The following appendix includes all results not included in **Chapter 6** but mentioned in the text.

Tables 1–5: Correlations between antibody responses to AMA1(3D7) and A4 schizont extract at each cross sectional bleed.

Tables 6–8: Antibody responses to AMA1(3D7) by age group and parasite status at each cross sectional bleed.

Tables 9–12: Antibody responses to AMA1(3D7) by reactivity to A4 schizont extract at each cross sectional bleed.

Tables 13–16: Antibody responses to AMA1(3D7) in the presence and absence of haemoglobinopathies.

Table 1 Correlations between antibody responses to AMA1(3D7) and schizont extract during the October 2002 cross sectional bleed.

	October 2002 Antibody responses				
	Schizont extract	Total IgG†	IgG1†	IgG3†	IgM†
Total IgG	0.7*	-	-	-	-
IgG1	0.64*	0.91*	-	-	-
IgG3	0.42*	0.56*	0.64*	-	-
IgM	0.13Δ	0.28*	0.39*	0.55*	-

Notes
Spearman rank correlation coefficients for IgG responses.
All samples tested.
† Antibody response to AMA1(3D7).
* *P*-value <0.001
Δ *P*-value <0.05

Table 2 Correlations between antibody responses to AMA1(3D7) and schizont extract during the May 2003 cross sectional bleed.

	May 2003 Antibody responses				
	Schizont extract	Total IgG†	IgG1†	IgG3†	IgM†
Total IgG	0.42*	-	-	-	-
IgG1	0.57*	0.67*	-	-	-
IgG3	0.45*	0.49*	0.69*	-	-
IgM	0.14Δ	0.31*	0.36*	0.55*	-

Notes
Spearman rank correlation coefficients for IgG responses.
All samples tested.
† Antibody response to AMA1(3D7).
* *P*-value <0.001
Δ *P*-value <0.05

Table 3 Correlations between antibody responses to AMA1(3D7) and schizont extract during the October 2003 cross sectional bleed.

	October 2003 Antibody responses				
	Schizont extract	Total IgG†	IgG1†	IgG3†	IgM†
Total IgG	0.73*	-	-	-	-
IgG1	0.66*	0.91*	-	-	-
IgG3	0.50*	0.62*	0.70*	-	-
IgM	0.15Δ	0.23*	0.36*	0.45*	-

Notes
Spearman rank correlation coefficients for IgG responses.
All samples tested.
† Antibody response to AMA1(3D7).
* *P*-value <0.001
Δ *P*-value <0.05

Table 4 Correlations between antibody responses to AMA1(3D7) and schizont extract during the May 2004 cross sectional bleed.

	May 2004 Antibody responses				
	Schizont extract	Total IgG†	IgG1†	IgG3†	IgM†
Total IgG	-0.17Δ	-	-	-	-
IgG1	-0.16Δ	0.88*	-	-	-
IgG3	-0.08	0.58*	0.70*	-	-
IgM	0.01	0.32*	0.47*	0.58*	-

Notes

Spearman rank correlation coefficients for IgG responses.

All samples tested.

† Antibody response to AMA1(3D7).

* *P*-value <0.001

Δ *P*-value <0.01

Table 5 Correlations between antibody responses to AMA1(3D7) and schizont extract during the October 2004 cross sectional bleed.

	October 2004 Antibody responses				
	Schizont extract	Total IgG†	IgG1†	IgG3†	IgM†
Total IgG	0.65*	-	-	-	-
IgG1	0.61*	0.88*	-	-	-
IgG3	0.31*	0.56*	0.66*	-	-
IgM	0.16Δ	0.29*	0.45*	0.60*	-

Notes

Spearman rank correlation coefficients for IgG responses.

All samples tested.

† Antibody response to AMA1(3D7).

* *P*-value <0.001

Δ *P*-value <0.01

Table 6 IgG1 responses to AMA1(3D7) by age group and parasite status.

IgG1		All Samples					Aparasitemic					Parasitemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]
May 2002[1]	N[2]	30	128	117	23		30	109	93	15		-	19	24	8	
	Median	0.01	0.01	0.16	0.16	**<0.001***	0.01	-0.01	0.11	0.29	**<0.001***	-	0.36	0.68	0.36	0.638
	IQR	(-0.03 - 0.19)	(-0.06 - 0.16)	(0.01 - 1.07)	(0.05 - 1.79)		(-0.03 - 0.19)	(-0.06 - 0.09)	(0 - 0.56)	(0.05 - 1.79)		-	(0.01 - 1.45)	(0.19 - 1.4)	(0.1 - 1.78)	
October 2002[1]	N[2]	37	110	108	36		36	105	99	31		1	5	9	5	
	Median	0.03	-0.04	0.08	0.08	**<0.001***	0.03	-0.04	0.08	0.34	**<0.001***	-0.04	0.35	0.28	0.24	0.406
	IQR	(-0.05 - 0.25)	(-0.09 - 0.09)	(-0.03 - 0.37)	(0.07 - 1.41)		(-0.05 - 0.26)	(-0.09 - 0.07)	(-0.03 - 0.35)	(0.04 - 1.33)		(-0.04 - -0.04)	(0.34 - 0.59)	(0.1 - 0.58)	(0.21 - 2.19)	
May 2003[1]	N[2]	27	102	105	51		26	93	88	40		1	9	17	11	
	Median	-0.02	-0.04	0.07	0.07	**<0.001***	-0.02	-0.06	0.05	0.38	**<0.001***	0.23	0.39	0.78	2.04	**0.004***
	IQR	(-0.09 - 0.46)	(-0.11 - 0.09)	(-0.04 - 0.58)	(0.08 - 2.04)		(-0.09 - 0.46)	(-0.11 - 0.05)	(-0.05 - 0.37)	(0.06 - 1.5)		(0.23 - 0.23)	(0.05 - 0.52)	(0.14 - 1.71)	(1.61 - 2.33)	
October 2003[1]	N[2]	23	103	110	58		23	93	91	45		-	10	18	13	
	Median	0.12	0.03	0.15	0.15	**<0.001***	0.12	0.02	0.11	0.32	**<0.001***	-	0.39	1.42	2.08	0.199
	IQR	(-0.01 - 0.2)	(-0.03 - 0.17)	(0.03 - 0.58)	(0.1 - 1.57)		(-0.01 - 0.2)	(-0.03 - 0.14)	(0.01 - 0.36)	(0.09 - 1.1)		-	(0.11 - 1.43)	(0.27 - 1.9)	(1.04 - 2.2)	
May 2004[1]	N[2]	21	93	86	79		21	88	84	69		-	5	2	10	
	Median	0.08	0.04	0.12	0.12	**<0.001***	0.08	0.04	0.12	0.31	**<0.001***	-	0.73	0.16	1.11	0.117
	IQR	(-0.01 - 0.2)	(0 - 0.16)	(0.04 - 0.32)	(0.08 - 1.15)		(-0.01 - 0.2)	(-0.01 - 0.14)	(0.04 - 0.32)	(0.08 - 0.86)		-	(0.19 - 1.14)	(0.1 - 0.22)	(0.8 - 1.55)	
October 2004[1]	N[2]	15	84	87	87		15	80	87	81		-	4	-	6	
	Median	0.04	-0.03	0.04	0.04	**<0.001***	0.04	-0.04	0.04	0.12	**<0.001***	-	0.47	-	0.61	0.670
	IQR	(-0.06 - 0.17)	(-0.08 - 0.06)	(-0.04 - 0.23)	(0.02 - 0.77)		(-0.06 - 0.17)	(-0.08 - 0.03)	(-0.04 - 0.23)	(0.01 - 0.75)		-	(0.28 - 0.67)	-	(0.21 - 1.16)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

* *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

Table 7 IgG3 responses to AMA1(3D7) by age group and parasite status.

IgG3		All Samples					Aparasitemic					Parasitemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]
May 2002[1]	N[2]	30	128	117	23		30	109	93	15		-	19	24	8	0.243
	Median	0.01	0.01	0.02	0.02	**<0.001***	0.01	0.01	0.02	0.03	**<0.001***	-	0.10	0.07	0.05	
	IQR	(0 - 0.03)	(0 - 0.04)	(0.01 - 0.1)	(0.02 - 0.1)		(0 - 0.03)	(0 - 0.03)	(0.01 - 0.07)	(0.02 - 0.1)		-	(0.05 - 0.2)	(0.01 - 0.11)	(0.03 - 0.08)	
October 2002[1]	N[2]	37	110	108	36		36	105	99	31		1	5	9	5	0.158
	Median	0.01	0.01	0.02	0.02	**0.002***	0.01	0.01	0.02	0.03	0.013*	0.01	0.02	0.07	0.25	
	IQR	(0.01 - 0.03)	(0 - 0.03)	(0.01 - 0.06)	(0.01 - 0.06)		(0.01 - 0.03)	(0 - 0.03)	(0.01 - 0.05)	(0.01 - 0.06)		(0.01 - 0.01)	(0.02 - 0.03)	(0.01 - 0.14)	(0.03 - 1.11)	
May 2003[1]	N[2]	27	102	105	51		26	93	88	40		1	9	17	11	0.344
	Median	0.00	0.00	0.02	0.02	**<0.001***	0.01	0.00	0.01	0.02	**<0.001***	-0.01	0.15	0.17	0.18	
	IQR	(-0.01 - 0.03)	(-0.01 - 0.02)	(0 - 0.05)	(0.01 - 0.13)		(-0.01 - 0.03)	(-0.01 - 0.02)	(0 - 0.04)	(0 - 0.08)		(-0.01 - -0.01)	(0 - 0.44)	(0.04 - 0.2)	(0.04 - 0.3)	
October 2003[1]	N[2]	23	103	110	58		23	93	91	45		-	10	18	13	0.540
	Median	0.02	0.02	0.02	0.02	0.090*	0.02	0.02	0.02	0.02	0.379	-	0.12	0.21	0.12	
	IQR	(0 - 0.04)	(0.01 - 0.06)	(0.01 - 0.07)	(0.01 - 0.11)		(0 - 0.04)	(0 - 0.04)	(0.01 - 0.04)	(0.01 - 0.08)		-	(0.09 - 0.4)	(0.07 - 0.41)	(0.05 - 0.15)	
May 2004[1]	N[2]	21	93	86	79		21	88	84	69		-	5	2	10	0.636
	Median	0.01	0.01	0.02	0.02	**<0.001***	0.01	0.01	0.02	0.03	**<0.001***	-	0.09	0.08	0.05	
	IQR	(0 - 0.02)	(0 - 0.03)	(0.01 - 0.04)	(0.01 - 0.07)		(0 - 0.02)	(0 - 0.02)	(0.01 - 0.04)	(0.01 - 0.06)		-	(0.05 - 0.2)	(0.02 - 0.15)	(0.02 - 0.23)	
October 2004[1]	N[2]	15	84	87	87		15	80	87	81		-	4	-	6	1.000
	Median	0.00	0.00	0.01	0.01	**0.002***	0.00	0.00	0.01	0.02	0.001*	-	0.09	-	0.12	
	IQR	(0 - 0.02)	(-0.01 - 0.02)	(0 - 0.03)	(0 - 0.04)		(0 - 0.02)	(-0.01 - 0.02)	(0 - 0.03)	(0 - 0.04)		-	(0.05 - 0.18)	-	(0.01 - 0.24)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

* *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

Table 8 IgM responses to AMA1(3D7) by age group and parasite status.

IgM		All Samples					Aparasitemic					Parasitemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[3]
May 2002[1]	N[2]	30	128	117	23		30	109	93	15		-	19	24	8	
	Median	0.02	0.02	0.05	0.05	**<0.001***	0.02	0.02	0.04	0.07	**<0.001***	-	0.06	0.06	0.08	0.578
	IQR	(0 - 0.05)	(0 - 0.06)	(0.02 - 0.08)	(0.02 - 0.16)		(0 - 0.05)	(0 - 0.05)	(0.02 - 0.08)	(0.02 - 0.13)		-	(0.03 - 0.1)	(0.02 - 0.11)	(0.04 - 0.17)	
October 2002[1]	N[2]	37	110	108	36		36	105	99	31		1	5	9	5	
	Median	0.02	0.02	0.03	0.03	**0.042***	0.02	0.02	0.03	0.04	0.119*	0.06	0.02	0.02	0.09	0.309
	IQR	(0.01 - 0.06)	(0.01 - 0.04)	(0.01 - 0.07)	(0.02 - 0.09)		(0.01 - 0.06)	(0.01 - 0.04)	(0.01 - 0.07)	(0.01 - 0.08)		(0.06 - 0.06)	(0.02 - 0.02)	(0.01 - 0.05)	(0.03 - 0.14)	
May 2003[1]	N[2]	27	102	105	51		26	93	88	40		1	9	17	11	
	Median	-0.01	-0.01	0.01	0.01	**<0.001***	0.00	-0.01	0.01	0.01	**0.009***	-0.04	0.00	0.03	0.10	0.049
	IQR	(-0.03 - 0.01)	(-0.03 - 0.03)	(-0.01 - 0.05)	(0 - 0.07)		(-0.03 - 0.01)	(-0.03 - 0.02)	(-0.01 - 0.05)	(-0.01 - 0.05)		(-0.04 - -0.04)	(0 - 0.05)	(-0.01 - 0.1)	(0.07 - 0.2)	
October 2003[1]	N[2]	23	103	110	58		23	93	91	45		-	10	18	13	
	Median	0.02	0.02	0.03	0.03	**0.043***	0.02	0.02	0.03	0.02	0.321	-	0.02	0.04	0.08	**0.032***
	IQR	(-0.01 - 0.04)	(0 - 0.05)	(0.01 - 0.05)	(0.01 - 0.08)		(-0.01 - 0.04)	(0 - 0.05)	(0 - 0.05)	(0 - 0.06)		-	(0 - 0.04)	(0.03 - 0.12)	(0.03 - 0.14)	
May 2004[1]	N[2]	21	93	86	79		21	88	84	69		-	5	2	10	
	Median	0.02	0.02	0.04	0.04	**0.017***	0.02	0.02	0.04	0.04	**0.024***	-	0.04	0.04	0.06	0.372
	IQR	(0.01 - 0.04)	(0.01 - 0.05)	(0.02 - 0.07)	(0.02 - 0.07)		(0.01 - 0.04)	(0.01 - 0.05)	(0.02 - 0.07)	(0.02 - 0.07)		-	(0.03 - 0.05)	(0.03 - 0.06)	(0.02 - 0.13)	
October 2004[1]	N[2]	15	84	87	87		15	80	87	81		-	4	-	6	
	Median	0.02	0.03	0.03	0.03	0.140	0.02	0.03	0.03	0.03	0.131	-	0.05	-	0.05	0.831
	IQR	(0 - 0.03)	(0.01 - 0.06)	(0.01 - 0.06)	(0.01 - 0.06)		(0 - 0.03)	(0 - 0.06)	(0.01 - 0.06)	(0.02 - 0.06)		-	(0.04 - 0.06)	-	(0 - 0.11)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA.

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

* *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Wilcoxon rank sum test (*P*-values not shown).

Table 9 Total IgG responses to AMA1(3D7) by reactivity to A4 schizont extract.

Total IgG		Reactivity to schizont extract			
		Low	Medium	High	*P*-value[3]
May 2002[1]	N[2]	102	100	101	
	Median	0.00	0.07	0.61	**<0.001**[‡*†]
	IQR	(-0.02 - 0.03)	(0.02 - 0.32)	(0.16 - 1.76)	
October 2002[1]	N[2]	99	98	97	
	Median	0.00	0.05	0.38	**<0.001**[‡*†]
	IQR	(-0.02 - 0.04)	(0 - 0.22)	(0.15 - 1.34)	
May 2003[1]	N[2]	97	98	95	
	Median	0.02	0.04	0.45	**<0.001**[‡†]
	IQR	(0 - 0.09)	(0 - 0.17)	(0.07 - 1.68)	
October 2003[1]	N[2]	96	95	95	
	Median	-0.01	0.08	0.96	**<0.001**[‡*†]
	IQR	(-0.02 - 0.03)	(0 - 0.23)	(0.18 - 1.97)	
May 2004[1]	N[2]	95	93	95	
	Median	0.06	0.05	0.02	**0.016**[‡]
	IQR	(0 - 0.74)	(-0.01 - 0.22)	(-0.02 - 0.18)	
October 2004[1]	N[2]	101	101	100	
	Median	-0.01	0.01	0.36	**<0.001**[‡*†]
	IQR	(-0.03 - 0.02)	(-0.01 - 0.07)	(0.11 - 0.95)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with high schizont reactivity (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with medium schizont reactivity (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with medium and children with high schizont reactivity (*P*-values not shown).

Table 10 IgG1 responses to AMA1(3D7) by reactivity to A4 schizont extract.

IgG1		Reactivity to schizont extract			
		Low	Medium	High	*P*-value[3]
May 2002[1]	N[2]	102	100	101	
	Median	-0.01	0.06	0.56	**<0.001**[‡*†]
	IQR	(-0.06 - 0.07)	(-0.01 - 0.25)	(0.14 - 1.5)	
October 2002[1]	N[2]	99	98	97	
	Median	-0.05	0.01	0.34	**<0.001**[‡*†]
	IQR	(-0.09 - 0.03)	(-0.05 - 0.17)	(0.11 - 1.26)	
May 2003[1]	N[2]	97	98	95	
	Median	-0.04	-0.02	0.58	**<0.001**[‡†]
	IQR	(-0.1 - 0.05)	(-0.09 - 0.19)	(0.14 - 1.76)	
October 2003[1]	N[2]	96	95	95	
	Median	0.01	0.11	0.77	**<0.001**[‡*†]
	IQR	(-0.04 - 0.1)	(0 - 0.28)	(0.18 - 1.83)	
May 2004[1]	N[2]	95	93	95	
	Median	0.14	0.10	0.07	**0.034**[‡]
	IQR	(0.04 - 0.81)	(0.02 - 0.31)	(0 - 0.26)	
October 2004[1]	N[2]	101	101	100	
	Median	-0.03	0.00	0.44	**<0.001**[‡*†]
	IQR	(-0.08 - 0.05)	(-0.05 - 0.09)	(0.08 - 1.09)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with high schizont reactivity (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with medium schizont reactivity (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with medium and children with high schizont reactivity (*P*-values not shown).

Table 11 IgG3 responses to AMA1(3D7) by reactivity to A4 schizont extract.

IgG3		Reactivity to schizont extract			
		Low	Medium	High	*P*-value[3]
May 2002[1]	N[2]	102	100	101	
	Median	0.01	0.01	0.07	**<0.001**[‡†]
	IQR	(-0.01 - 0.02)	(0 - 0.03)	(0.03 - 0.17)	
October 2002[1]	N[2]	99	98	97	
	Median	0.01	0.02	0.04	**<0.001**[‡†]
	IQR	(0 - 0.02)	(0.01 - 0.04)	(0.02 - 0.1)	
May 2003[1]	N[2]	97	98	95	
	Median	0.00	0.00	0.04	**<0.001**[‡†]
	IQR	(-0.01 - 0.02)	(-0.01 - 0.03)	(0.01 - 0.17)	
October 2003[1]	N[2]	96	95	95	
	Median	0.01	0.02	0.08	**<0.001**[‡†]
	IQR	(0 - 0.03)	(0.01 - 0.04)	(0.02 - 0.18)	
May 2004[1]	N[2]	95	93	95	
	Median	0.02	0.01	0.01	0.128[*]
	IQR	(0 - 0.05)	(0 - 0.03)	(0 - 0.05)	
October 2004[1]	N[2]	101	101	100	
	Median	0.00	0.00	0.03	**<0.001**[‡†]
	IQR	(-0.01 - 0.02)	(-0.01 - 0.02)	(0.01 - 0.06)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with high schizont reactivity (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with medium schizont reactivity (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with medium and children with high schizont reactivity (*P*-values not shown).

Table 12 IgM responses to AMA1(3D7) by reactivity to A4 schizont extract.

IgM		Reactivity to schizont extract			
		Low	Medium	High	*P*-value[3]
May 2002[1]	N[2]	102	100	101	
	Median	0.03	0.03	0.06	**<0.001**[‡†]
	IQR	(0 - 0.06)	(0.01 - 0.07)	(0.02 - 0.09)	
October 2002[1]	N[2]	99	98	97	
	Median	0.02	0.02	0.03	0.082[†]
	IQR	(0 - 0.05)	(0.01 - 0.05)	(0.01 - 0.09)	
May 2003[1]	N[2]	97	98	95	
	Median	0.01	0.00	0.02	**<0.001**[‡†]
	IQR	(-0.01 - 0.04)	(-0.03 - 0.02)	(-0.01 - 0.07)	
October 2003[1]	N[2]	96	95	95	
	Median	0.02	0.02	0.03	**0.051**[‡]
	IQR	(0 - 0.04)	(0 - 0.05)	(0.01 - 0.08)	
May 2004[1]	N[2]	95	93	95	
	Median	0.02	0.03	0.03	0.950
	IQR	(0.01 - 0.07)	(0.02 - 0.05)	(0.02 - 0.06)	
October 2004[1]	N[2]	101	101	100	
	Median	0.03	0.03	0.04	**0.032**[‡†]
	IQR	(0.01 - 0.05)	(0.01 - 0.05)	(0.02 - 0.08)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with high schizont reactivity (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with low and children with medium schizont reactivity (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children with medium and children with high schizont reactivity (*P*-values not shown).

Table 13 Total IgG responses to AMA1(3D7) in the presence or absence of hemoglobinopathies.

Total IgG		Sickle trait			Alpha-Thalassemia			
		HbAA	HBAS	*P*-value[3]	Norm	HbAA	HBAS	*P*-value[3]
May 2002[1]	N[2]	258	41		93	152	52	
	Median	0.07	0.04	0.162	0.06	0.08	0.04	0.357
	IQR	(0 - 0.6)	(0 - 0.15)		(-0.01 - 0.59)	(0.01 - 0.51)	(0.01 - 0.21)	
October 2002[1]	N[2]	258	34		92	138	54	
	Median	0.06	0.05	0.368	0.05	0.09	0.02	0.051[*†]
	IQR	(0 - 0.33)	(0 - 0.19)		(0 - 0.31)	(0 - 0.38)	(-0.01 - 0.14)	
May 2003[1]	N[2]	246	37		90	137	52	
	Median	0.08	0.02	**0.02**	0.06	0.08	0.03	**0.02**[†]
	IQR	(0.01 - 0.6)	(-0.01 - 0.24)		(0 - 0.5)	(0.01 - 0.65)	(-0.01 - 0.13)	
October 2003[1]	N[2]	246	38		97	136	52	
	Median	0.08	0.07	0.843	0.10	0.08	0.02	0.07[*]
	IQR	(-0.01 - 0.46)	(0.01 - 0.19)		(0 - 0.74)	(0 - 0.45)	(-0.01 - 0.13)	
May 2004[1]	N[2]	231	35		88	124	49	
	Median	0.04	0.04	0.47	0.07	0.04	0.00	**0.032**[*†]
	IQR	(-0.01 - 0.36)	(-0.01 - 0.14)		(-0.01 - 0.6)	(-0.01 - 0.36)	(-0.01 - 0.08)	
October 2004[1]	N[2]	232	38		92	126	50	
	Median	0.02	0.04	0.728	0.03	0.04	0.02	0.721
	IQR	(-0.01 - 0.22)	(-0.02 - 0.18)		(-0.01 - 0.27)	(-0.02 - 0.24)	(-0.01 - 0.18)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Wilcoxon rank test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children heterozygous for α-thalassemia (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children homozygous for α-thalassemia (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children heterozygous and homozygous for α-thalassemia (*P*-values not shown).

Table 14 IgG1 responses to AMA1(3D7) in the presence or absence of hemoglobinopathies.

IgG1		Sickle trait			Alpha-Thalassemia			
		HbAA	HBAS	*P*-value[3]	Norm	HbAA	HBAS	*P*-value[4]
May 2002[1]	N[2]	258	41		93	152	52	
	Median	0.08	0.08	0.54	0.05	0.09	0.05	0.343
	IQR	(-0.03 - 0.49)	(-0.01 - 0.19)		(-0.03 - 0.62)	(-0.01 - 0.46)	(-0.04 - 0.18)	
October 2002[1]	N[2]	258	34		92	138	54	
	Median	0.03	0.03	0.365	0.03	0.05	-0.01	0.132[†]
	IQR	(-0.05 - 0.3)	(-0.06 - 0.18)		(-0.04 - 0.33)	(-0.05 - 0.33)	(-0.07 - 0.15)	
May 2003[1]	N[2]	246	37		90	137	52	
	Median	0.05	0.01	0.258	0.05	0.07	-0.01	0.094[†]
	IQR	(-0.07 - 0.58)	(-0.08 - 0.17)		(-0.06 - 0.46)	(-0.06 - 0.63)	(-0.11 - 0.18)	
October 2003[1]	N[2]	246	38		97	136	52	
	Median	0.12	0.11	0.638	0.15	0.13	0.06	**0.033**[*]
	IQR	(0 - 0.48)	(0.01 - 0.28)		(0.05 - 0.63)	(0.01 - 0.45)	(-0.01 - 0.19)	
May 2004[1]	N[2]	231	35		88	124	49	
	Median	0.11	0.12	0.606	0.14	0.12	0.06	0.063[*]
	IQR	(0.02 - 0.48)	(0.03 - 0.22)		(0.03 - 0.73)	(0.02 - 0.5)	(0.02 - 0.16)	
October 2004[1]	N[2]	258	41		92	126	50	
	Median	0.08	0.08	0.54	0.08	0.04	0.02	0.225
	IQR	(-0.03 - 0.49)	(-0.01 - 0.19)		(-0.03 - 0.36)	(-0.05 - 0.36)	(-0.05 - 0.22)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Wilcoxon rank test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children heterozygous for α-thalassemia (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children homozygous for α-thalassemia (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children heterozygous and homozygous for α-thalassemia (*P*-values not shown).

Table 15 IgG3 responses to AMA1(3D7) in the presence or absence of hemoglobinopathies.

IgG3		Sickle trait			Alpha-Thalassemia			
		HbAA	HBAS	*P*-value[3]	Norm	HbAA	HBAS	*P*-value[4]
May 2002[1]	N[2]	258	41		93	152	52	
	Median	0.02	0.02	0.912	0.02	0.02	0.01	0.745
	IQR	(0 - 0.07)	(0 - 0.05)		(0 - 0.07)	(0 - 0.06)	(0 - 0.05)	
October 2002[1]	N[2]	258	34		92	138	54	
	Median	0.02	0.02	0.925	0.02	0.02	0.02	0.474
	IQR	(0.01 - 0.05)	(0 - 0.03)		(0.01 - 0.04)	(0.01 - 0.05)	(0 - 0.03)	
May 2003[1]	N[2]	246	37		90	137	52	
	Median	0.01	0.01	0.255	0.01	0.01	0.01	0.363
	IQR	(0 - 0.04)	(-0.01 - 0.03)		(0 - 0.04)	(0 - 0.05)	(-0.01 - 0.03)	
October 2003[1]	N[2]	246	38		97	136	52	
	Median	0.02	0.03	0.758	0.03	0.02	0.02	0.191
	IQR	(0.01 - 0.07)	(0.01 - 0.07)		(0.01 - 0.08)	(0.01 - 0.06)	(0.01 - 0.05)	
May 2004[1]	N[2]	231	35		88	124	49	
	Median	0.02	0.01	0.849	0.02	0.02	0.01	0.536
	IQR	(0 - 0.04)	(0 - 0.04)		(0 - 0.05)	(0 - 0.04)	(0 - 0.04)	
October 2004[1]	N[2]	232	38		92	126	50	
	Median	0.01	0.02	0.158	0.01	0.01	0.01	0.306
	IQR	(0 - 0.03)	(0 - 0.05)		(0 - 0.03)	(0 - 0.03)	(0 - 0.03)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Wilcoxon rank test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[‡] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children heterozygous for α-thalassemia (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children homozygous for α-thalassemia (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children heterozygous and homozygous for α-thalassemia (*P*-values not shown).

Table 16 IgM responses to AMA1(3D7) in the presence or absence of hemoglobinopathies.

IgM		Sickle trait			Alpha-Thalassemia			
		HbAA	HBAS	*P*-value[3]	Norm	HbAA	HBAS	*P*-value[4]
May 2002[1]	N[2]	258	41		93	152	52	
	Median	0.03	0.05	0.147	0.03	0.04	0.04	0.672
	IQR	(0.01 - 0.07)	(0.02 - 0.09)		(0.01 - 0.07)	(0.01 - 0.07)	(0.01 - 0.09)	
October 2002[1]	N[2]	258	34		92	138	54	
	Median	0.03	0.02	0.534	0.03	0.03	0.02	0.345
	IQR	(0.01 - 0.06)	(0 - 0.05)		(0.01 - 0.06)	(0 - 0.05)	(0.01 - 0.05)	
May 2003[1]	N[2]	246	37		90	137	52	
	Median	0.01	0.00	0.538	0.01	0.01	-0.01	**0.005**[*†]
	IQR	(-0.02 - 0.05)	(-0.02 - 0.04)		(-0.02 - 0.05)	(-0.01 - 0.05)	(-0.03 - 0.02)	
October 2003[1]	N[2]	246	38		97	136	52	
	Median	0.02	0.03	0.284	0.02	0.02	0.02	0.801
	IQR	(0 - 0.05)	(0 - 0.07)		(0 - 0.06)	(0 - 0.05)	(0 - 0.04)	
May 2004[1]	N[2]	231	35		88	124	49	
	Median	0.03	0.04	0.356	0.03	0.03	0.02	0.508
	IQR	(0.01 - 0.06)	(0.02 - 0.09)		(0.02 - 0.06)	(0.01 - 0.07)	(0.01 - 0.05)	
October 2004[1]	N[2]	232	38		92	126	50	
	Median	0.03	0.04	0.58	0.03	0.03	0.03	0.371
	IQR	(0.01 - 0.06)	(0.01 - 0.06)		(0.02 - 0.07)	(0.01 - 0.06)	(0.02 - 0.06)	

Notes

[1] Cross sectional bleed.

[2] Number of samples tested by ELISA

[3] *P*-values calculated using a Wilcoxon rank test (*P*-values ≤ 0.05 indicated in bold type).

[4] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children heterozygous for α-thalassemia (*P*-values not shown).

[*] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between normal children and children homozygous for α-thalassemia (*P*-values not shown).

[†] Significant difference ($P \leq 0.05$ Wilcoxon rank test) in median IgG levels between children heterozygous and homozygous for α-thalassemia (*P*-values not shown).